MOLECULAR MECHANISMS CONTROLLING EPITHELIAL TO MESENCHYMAL TRANSITION AND CELL INVASION IN DEVELOPMENT AND CANCER

by

Laura Jimenez

A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Oncological Sciences

The University of Utah

December 2015

Copyright © Laura Jimenez 2015

All Rights Reserved

The University of Utah Graduate School

STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Laura Jimenez**

has been approved by the following supervisory committee members:

Rodney Stewart	, Chair	**10/5/15** Date Approved
Jody Rosenblatt	, Member	**10/5/15** Date Approved
Douglas Grossman	, Member	**10/5/15** Date Approved
David Grunwald	, Member	**10/5/15** Date Approved
Sheri Holmen	, Member	**10/5/15** Date Approved

and by **Bradley Cairns**, Chair/Dean of the Department/College/School of **Oncological Sciences**

and by David B. Kieda, Dean of The Graduate School.

ABSTRACT

The conversion of epithelial cells into migratory and invasive mesenchymal cells has been established as a fundamental step in the metastatic cascade. In cancer cells the induction of an epithelial-to-mesenchymal transition (EMT) awards invasive properties that allow dissemination from the primary tumor, and promotes acquisition of stem-cell-like properties and prosurvival mechanisms that contribute to therapeutic resistance, relapse, and decreased overall survival. Identifying inhibitors that block EMT is of great therapeutic interest to prevent disseminated tumor cells and target dormant cancer stem cells. Efforts to rationally target EMT in the clinic have been impeded by our incomplete understanding of the molecular mechanisms regulating EMT in normal and cancerous cells.

Here we develop a zebrafish *in vivo* EMT reporter for rapid screening of small molecules called *Tg(snai1b:GFP),* which labels snai1b-expressing cells during development undergoing EMT from the dorsal neural tube to form neural crest (NC). We identified a multi-kinase inhibitor, called TP-0903, that potently blocked EMT, migration, and differentiation of dorsal neural tube progenitors by activating retinoic acid (RA) signaling. We show for the first time that RA directly controls EMT of NC cells *in vivo*. Our findings designate RA as an attractive therapeutic strategy to antagonize EMT-dependent pathways and stem-cell-like

properties to eliminate residual cancer stem cells and metastatic disease.

TP-0903 was rationally designed to target AXL RTK, a key oncogenic target that promotes EMT and metastasis. In NC derived cancers like melanoma, AXL can mediate invasion and promote resistance to targeted therapies *in vitro*. We hypothesize AXL promotes malignant melanoma by inducing EMT of cancer cells *in vivo*. To test this we generated a zebrafish melanoma model overexpressing AXL. We found AXL accelerated melanocyte transformation, tumor onset, growth and invasion. Additionally, we found that AXL did not activate EMT programs *in vivo*, despite numerous studies implicating AXL as a driver of EMT *in vitro.* Nevertheless these studies specify AXL as a driver of melanoma and establish an *in vivo* zebrafish model of AXL dependent melanoma invasion. This preclinical model will help determine the AXL dependent mechanisms driving malignant melanoma and evaluate effective AXL inhibitors to treat melanoma patients.

To my parents, my greatest source of inspiration.

“Above all, don't fear difficult moments.
The best comes from them.”
Rita Levi-Montalcini

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

First, I would like to thank my PhD mentor, Dr. Rodney Stewart for training me to be a rigorous scientist and supporting my career development. Rod has provided me with a stimulating and challenging environment to develop scientifically and intellectually, and for that I am grateful.

I am also thankful to my thesis committee members: Dr. Jody Rosenblatt, Dr. David Grunwald, Dr. Doug Grossman, Dr. Sheri Holmen, and Dr. David Bearss who have challenged me to think critically and have been very generous with their time and feedback. Additionally, I thank the Multidisciplinary Cancer Research Training Program, led by Dr. Don Ayer, for support through funding and career development opportunities that have been integral to my training.

During my graduate school studies, I have been surrounded by incredible colleagues and friends. I am thankful to all current and past members of the Stewart Lab for their daily enthusiasm and excitement for science. In particular I thank Kate Modzelewska, Shelly Sorrells, Elena Boer, and Monique Morrison who have provided constant feedback on my project and significant technical support. I am also thankful to my undergraduate research assistant Heather for her help screening and generating zebrafish mutants. Outside the lab my core group of friends have been a crucial part of my success, especially Peter, Ashot, Tanya, Gisela, Andres, and Marina who have provided constant motivation and

offered many needed diversions.

Lastly, I am grateful to my entire family, and my boyfriend Matt, who has provided an endless source of encouragement and motivation throughout my entire educational journey.

CHAPTER 1

INTRODUCTION

Metastasis

Metastasis is the most difficult stage of cancer to treat and is the leading cause of death in solid tumor patients. Tumor cells become metastatic by acquiring genetic and epigenetic alterations that confer the ability to escape the primary tumor, survive passage in the bloodstream, extravagate through the capillary endothelium and colonize distant sites (Fidler, 2003). This multistep process depends on complex biological and biochemical changes in the cell as well as in the surrounding stroma that are still not completely understood. Understanding the molecular mechanisms that promote metastasis is necessary to develop targeted therapies against this disease. Advances in the molecular profiling of cancer using genomic approaches have identified genes necessary for the successful execution of the metastatic cascade (Weigelt et al., 2005). These findings implicate numerous pathways in metastatic progression and have increased the feasibility of identifying stages of metastasis that may be susceptible to therapeutic intervention.

Epithelial-to-mesenchymal transition

One stage of metastasis that has recently emerged at the forefront of cancer research is the epithelial-to-mesenchymal transition (EMT). EMT is a highly conserved molecular and cellular program that facilitates the conversion of epithelial cells into motile and invasive mesenchymal cells. EMT is essential during embryogenesis, wound healing, and contributes pathologically to fibrosis and cancer metastasis (Polyak and Weinberg, 2009a). Induction of EMT causes

epithelial cells to lose apical-basal polarity as tight junctions and cell-cell junctions are disassembled (Huang et al., 2012). In addition, cytoskeletal proteins are rearranged to achieve a front-back end polarity necessary for directional migration, and extracellular matrix (ECM) degrading enzymes are produced and secreted to destroy the underlying basement membrane (Lamouille et al., 2014). These changes transition an epithelial cell from a cuboidal epithelial shape to a more spindle mesenchymal shape and allow dissociation from neighboring cells and invasion into surrounding tissue (Hay, 1995).

In addition to changes in cell shape and acquisition of motility, EMT promotes fundamental alterations in the gene expression profiles of cells. During the transdifferentiation of cells, expression of genes that mediate the epithelial phenotype are repressed including cytokeratins, epithelial cell polarity proteins, and adherens junction proteins like E-cadherin (Huang et al., 2012; Thiery and Sleeman, 2006). Concurrently, expression of genes mediating the mesenchymal phenotype is induced, including components of the mesenchymal cytoskeleton, vimentin, mesenchymal adherens junction proteins like N-cadherin, proteases, integrins, and ECM proteins (Thiery and Sleeman, 2006). The execution of EMT is dependent on these gene-expression changes and is highly coordinated by a network of intracellular and extracellular signals that converge on a set of key transcription factors (TF) that function as the master regulators of EMT (Kalluri and Weinberg, 2009). Key findings from both development and cancer have provided significant advances in identifying the transcription factors, adhesion molecules, and signaling pathways that mediate EMT (Lim and Thiery, 2012).

Our understanding of EMT has increased significantly and it is now clear that EMT programs promote cancer cell survival, stemness, inflammation, and immunity (Nieto, 2011). These advances have established the EMT programs as major therapeutic target to treat cancer.

EMT in development

EMT was initially described in early phases of embryonic development when tissue remodeling and cell migration are critical (Trelstad et al., 1967). During embryonic development, EMT is a crucial process during implantation of the embryo into the uterus, gastrulation to form the three germ layers, and neural crest (NC) formation (Lim and Thiery, 2012). The parallels between developmental EMT and pathogenic EMT validate the embryo as an excellent model to study the molecular and cellular mechanisms driving EMT. Several key findings from embryonic development have helped to assemble our current understanding of EMT and have allowed the identification of EMT programs that are exploited by cancer cells (Thiery et al., 2009).

EMT is required during gastrulation when individual cells dissociate from the ectoderm and migrate towards the center of the embryo to form the mesoderm. Studies during gastrulation have identified numerous signaling pathways responsible for initiating EMT. Primary inducers of EMT during gastrulation include Wnt, fibroblast growth factor (FGF), and family members of transforming growth factor β (TGFβ) including Nodal, and vg1 (Heisenberg and Solnica-Krezel, 2008). In response to EMT inducing signals, gastrulating cells

upregulate the key transcription factors (TFs) Snail and Twist that function as repressors of E-cadherin and are required for gastrulation. Snail is part of a family of a DNA binding zinc finger proteins that act mainly as transcriptional repressors. Loss of function studies in sea urchins, flies, chicks, and mice reveal a highly conserved role for Snail during gastrulation (Carver et al., 2001; Grosshans and Wieschaus, 2000; Nieto et al., 1994; Wu et al., 2007). In all species tested, embryos deficient for Snail fail to gastrulate due to the inability of mesoderm to downregulate E-Cadherin (Carver et al., 2001; Grosshans and Wieschaus, 2000; Nieto et al., 1994; Wu et al., 2007). These findings underlie a functional role of Snail and its downstream effectors in promoting changes in cell shape, cell adhesion and cell movements to execute the EMT program. Further studies support a role for Snail in facilitating degradation of the underlying basement membrane by activating metalloproteases and repressing components of laminin to promote invasion into the ECM (Haraguchi et al., 2008; Jorda et al., 2005).

Twist is also a TF induced by primary EMT signals during gastrulation. Twist is a basic helix-loop-helix transcription factor that indirectly represses E-cadherin and is essential for promoting EMT in invertebrates. In sea urchins, loss of Twist inhibits gastrulation (Wu et al., 2008). In flies, Twist orchestrates rapid changes in cell shape by interacting with the transmembrane protein T48 and recruiting Rho GTP-exchange factor and cytoskeletal remodeler, RhoGEF2, to adherens junctions (Kolsch et al., 2007). In vertebrates, the T48 protein is not conserved and Twist is not required for gastrulation, indicating that Snail may

compensate for Twist and accomplish all of these functions. Other critical repressors of E-cadherin that have been identified during gastrulation include p38-MAPK, p38 interacting protein (p38-IP) and the FERM (EPB4.1L5) which directly repress E-cadherin protein levels (Thiery et al., 2009).

NC development represents one of the key EMT programs that occurs during embryonic development. NC progenitors undergo EMT from the dorsal neural epithelium, migrate extensively throughout the embryo and differentiate into a variety of cell types, including neurons and glia in the peripheral nervous system, connective tissue and bone that make up craniofacial structures, and melanocytes (Le Douarin and Kalcheim, 1999). The gene regulatory network (GRN) operating during neural crest EMT is induced by a number of growth factors that specify the neural crest territory during NC induction. The combined action of growth factors TGFβ, BMP, FGF and Wnt induce expression and/or stability of canonical EMT transcription factors Snail1/2, Twist1 and Zeb1/2 (Lee et al., 2013; Powell et al., 2013). However, direct evidence that any of these growth factors directly control EMT is lacking, and the functional loss of individual EMT TFs does not result in the dramatic EMT defects observed during gastrulation (Murray and Gridley, 2006; Tang et al., 2010). For instance, Snail is required for gastrulation across multiple species but in mice loss of *Snail1* and *Snail2* does not disrupt EMT and neural crest formation (Murray and Gridley, 2006). These observations suggest that an extraordinary degree of cooperation and plasticity exists within the NC EMT GRN. Indeed, many regulatory loops among NC EMT inducers and TFs have been identified which enable

compensation after loss of a single regulator (Thiery and Sleeman, 2006). Our understanding of the GRNs operating during embryogenesis remains incomplete, partly due to the difficulty in labeling and observing the transient nature of EMT in a whole animal. Future studies will benefit from new transgenic animal models that label cells for visualization of EMT *in vivo* and isolation for genome wide approaches.

The NC provides a unique opportunity to study the complexity of signaling networks regulating EMT as there is a close resemblance between the formation of the vertebrate NC and the stages of metastasis. During both processes, cells delaminate from the primary tissue by undergoing an epithelial-mesenchymal transition (EMT), actively migrate, colonize distant target sites and finally differentiate into a secondary tumor or a NC derivative (Theveneau and Mayor, 2012). A more comprehensive understanding of the molecular and cellular regulators of NC EMT will provide insight into the developmental programs that can be exploited by cancer cells to promote invasion and metastasis. Not surprisingly genes controlling NC development are aberrantly activated during metastasis of several tumors including NC derived and non-NC derived tumors (Gupta et al., 2005; Yang et al., 2004). For this reason, the NC serves as an excellent model to better understand the invasion mechanisms of all cancers especially NC derived cancers like melanoma.

EMT in cancer

Local invasion is a necessary first step in metastatic dissemination; the induction of EMT in cancer cells enables invasive and migratory behaviors that contribute to this step in tumor progression (Hanahan and Weinberg, 2011). Carcinoma cells undergoing EMT have been observed at the invasive edge of primary tumors (Hlubek et al., 2007). There is a strong resemblance in the cellular reorganization exhibited by invasive carcinoma cells and embryonic cells undergoing EMT. In cancer cells loss of E-cadherin also plays a dominant role in influencing EMT and the malignant behavior of epithelial cancer cells. Inactivation of E-cadherin in cancer cells through transcriptional repression, promoter methylation, or functional mutations results in changes in cellular morphology, motility and ability to invade into surrounding tissue (Jeanes et al., 2008). In cancer cells, active transcriptional repression of E-cadherin is achieved through induction of EMT-associated TFs required in embryonic development (Batlle et al., 2000; Cano et al., 2000; Peinado et al., 2004). For example, in cancer cells expression of Snail represses E-cadherin expression and induces EMT in epithelial tumors similar to its role in development (Batlle et al., 2000; Cano et al., 2000). In human tumor samples, expression of Snail and Twist significantly correlates with disease relapse and poor survival in patients with breast, colorectal, ovarian, and melanoma (Hoek et al., 2004; Puisieux et al., 2014; Taube et al., 2010). Additionally, high expression of EMT TFs is correlated with high histological tumor grade and a subtype of invasive lobular breast carcinoma, a highly infiltrating tumor type associated with loss of E-cadherin

expression (Yang et al., 2004). The similarities between EMT signaling during embryogenesis and tumor development provide evidence that cancer cells reactivate developmental EMT programs in order to acquire invasive and migratory characteristics that promote metastatic dissemination (Gupta et al., 2005; Yang et al., 2004).

TGFB signaling

The signaling pathways regulating EMT during cancer are not completely defined but are controlled by similar signaling molecules that execute EMT during embryogenesis. For example, TGFβ is a primary inducer of EMT in cancer cells and is a prerequisite for cancer cell invasion and dissemination (Katsuno et al., 2013; Lamouille et al., 2014). Activation of TGFβ suppresses E-cadherin expression and induces the cellular conversion of cancer cells into a spindle cell shape (Portella et al., 1998). Suppression of E-cadherin in TGFβ stimulated cells is facilitated partly by increased expression and activity of Snail. TGFβ activates SMAD to induce transcription of Snail and to cooperate with Snail in a co-repressor complex that increases Snail activity (Hoot et al., 2008; Vincent et al., 2009). Expression and activity of other EMT TFs like Zeb and Twist are also induced through TGFβ in cancer cells (Kang et al., 2003; Shirakihara et al., 2007). TGFβ induces expression of Zeb through MAP Kinase dependent signaling (Shirakihara et al., 2007) and enhances activity and expression of Twist by indirectly repressing ID1 expression (Kang et al., 2003). Additionally, TGFβ can directly contribute to the disassembly of epithelial cells by inducing RHOA

degradation at tight junctions (Ozdamar et al., 2005) and promotes a mesenchymal switch by directly activating expression of mesenchymal genes like fibronectin and vimentin (Kaimori et al., 2007; Nawshad et al., 2007). These findings provide evidence that TGFβ signaling plays a central role in mediating EMT in cancer cells.

Studies in cancer cells have enabled the identification of many downstream mediators of TGFβ signaling that are required for TGFβ-induced EMT. For example, signaling transduced through RHO-like GTPases, PI3K, mTORC, and MAPK pathways can facilitate TGFβ induced EMT (Moustakas and Heldin, 2005). TGFβ promotes cytoskeletal changes that enable invasion and motility by inducing expression of GEFs and activation of ROCK and LIMK to stimulate RHOA activation (Bhowmick et al., 2001; Shen et al., 2001; Vardouli et al., 2005). Activation of GTPases like RHOA are required in TGFβ -induced EMT to regulate actin dynamics and reorganization which drive formation of lamellipodia and filopodia that help direct motility (Ridley, 2011). Activation of the mTORC pathway through TGFβ induced PI3K-AKT signaling is also essential to promote invasion and migration of cancer cells undergoing EMT (Bakin et al., 2000; Lamouille et al., 2012; Lamouille and Derynck, 2007). Additionally TGFβ can stimulate PI3K-AKT and MAPK signaling to increase expression and stabilization of Snail (Julien et al., 2007; Marchetti et al., 2008). These studies highlight the ability of TGFβ to activate several pathways and suggest the existence of signaling cooperation and convergence of these pathways on common EMT targets. It should be noted that the downstream mediators of

TGFβ have mainly been identified from studies in cell culture and further work is required to test the necessity of these signals *in vivo*. In the future, complementary *in vivo* approaches that allow labeling and observation of EMT in living animals are needed to identify effective inducers of EMT *in vivo*.

MicroRNAs

The advancement of novel molecular mechanisms regulating EMT has been facilitated by studies in cancer research. Among these, microRNAs have emerged as important inducers and suppressors of the EMT program. For example, miR-1 and miR-200 directly suppress EMT by inhibiting Snail expression and the miR-200 family along with miR-205 function similarly by repressing expression of Zeb (Gregory et al., 2008; Liu et al., 2013). Additional microRNAs that induce EMT have also been identified, such as miR-9, which directly suppresses E-cadherin expression (Ma et al., 2010). These studies illustrate the crucial role of microRNAs in dictating the progression of EMT.

Alternative splicing

Intriguingly, extensive changes in splicing patterns have been documented to accompany the transdifferentiation of epithelial cells into mesenchymal cells. Several studies show that primary inducers of EMT can promote a shift in splicing patterns and highlight a key role of splicing mediators in controlling EMT (Shapiro et al., 2011; Shirakihara et al., 2011). The importance of alternative splicing in EMT has been demonstrated by epithelial splicing variant 1 and 2

(ESPR1 and ESPR2), which mediate a splicing program that is suppressed during EMT (Taube et al., 2010; Warzecha et al., 2010). Together, these studies established alternative splicing as a novel mechanism to control EMT.

Epigenetic regulators

Epigenetic control of EMT has also been implicated as a novel mechanism in cancer cells. Aberrant DNA methylation patterns affecting EMT inducers and effectors during carcinoma progression have been identified in a mouse model of skin carcinogenesis (Fraga et al., 2004). Epigenetic modification by SIN3A, a histone deacetylase, promotes EMT by cooperating with Snail to repress E-cadherin expression (Herranz et al., 2008; Peinado et al., 2004). In contrast, epigenetic modifiers also inhibit EMT and cancer invasion by repressing Snail expression (Fujita et al., 2003). These studies provide new insights into the diverse molecular mechanisms regulating EMT and add a new level of regulation between the epithelial and mesenchymal states.

RTK signaling

Induction of EMT can also be accomplished by activation of receptor tyrosine kinases (RTKs). Similar to TGFβ signaling, RTKs can induce EMT by stimulating the activation of multiple signaling pathways including PI3K-AKT, MAPK, and JNK pathways (Lamouille et al., 2014). Numerous RTKs activate EMT in cancer cell lines, which highlight the importance of the downstream signal transducers PI3K and MAPK in the regulation of EMT. For example, c-MET RTK

can stimulate the induction of EMT through increasing expression of Snail (Doehn et al., 2009). The induction of Snail expression by c-MET requires activation of a key effector of the MAPK pathway (Doehn et al., 2009). Activating oncogenic mutations in RAF and RAS also induce EMT by stabilizing Snail proteins and RHO-GTPases, which further supports the role of MAPK signaling in promoting motile and invasive behavior of carcinoma cells (Makrodouli et al., 2011). Remarkably, the intracellular signals stimulated by most RTKs converge on the induction and activation of EMT TFs and repression of epithelial adhesion junctions. Such is the case for fibroblast growth factor receptor (FGFR), which induces EMT in cancer cells by dissociating desmosomes and promoting expression of Snail (Billottet et al., 2008; Savagner et al., 1997; Valles et al., 1996). In colon adenocarcinomas, platelet-derived growth factor receptor (PDGFR) induces EMT through the dissolution of adherens junctions, which promotes the nuclear translocation of β-catenin through a WNT-independent pathway (Yang et al., 2006). Additionally, epidermal growth factor receptor (EGFR) can promote EMT by inducing endocytosis of E-cadherin and expression of Snail and Twist (Lo et al., 2007; Lu et al., 2003).

Together, the studies described above emphasize the diversity of signals that promote EMT and demonstrate that an intricate network of signals work in parallel to activate common EMT targets. Furthermore, these findings provide rational therapeutic targets that can be investigated as antimetastatic agents.

AXL signaling in EMT and cancer

The RTK AXL has been implicated in EMT induction and is currently being investigated as a therapeutic target for the treatment of metastatic cancers (Gjerdrum et al., 2010; Linger et al., 2010). AXL is part of the TAM (Tyro3, Axl, Mer) family of RTKs characterized by an adhesion molecule-like domain in the extracellular region that consists of two fibronectin type 3 motifs and two immunoglobulinlike repeats. Activation of AXL by its ligand Gas6 or AXL overexpression stimulates several signaling pathways including PI3K-AKT, mTORC, NF-kB, STAT3 and MAPK signaling (Hafizi and Dahlback, 2006). AXL regulates a variety of cellular processes including cell proliferation, cell survival, cell adhesion and migration (Linger et al., 2008). Overexpression of AXL has been reported in several types of leukemias and solid tumors and its expression independently predicts poor prognosis in several cancers (Linger et al., 2008). Several studies have demonstrated that AXL signaling contributes to cancer progression by mediating cell survival, invasion, migration, metastasis and resistance to standard chemotherapies and RTK inhibitors (Linger et al., 2010).

Recent studies have revealed that AXL can induce EMT *in vitro* and is highly associated with the mesenchymal phenotype in human cancer cells (Asiedu et al., 2014; Gjerdrum et al., 2010; Wilson et al., 2014). Induction of EMT by overexpressing EMT TFs Twist, Zeb2, Snail, and Slug increases expression and activation of AXL, which promotes EMT-associated cytoskeletal and morphological changes (Gjerdrum et al., 2010; Holland et al., 2010). Interestingly, AXL expression is required to maintain Snail, Slug, and Twist

expression in pancreatic adenocarcinoma cells (Koorstra et al., 2009). Together these studies indicate that AXL is involved in a positive feedback loop that promotes and sustains the mesenchymal phenotype of cancer cells. Additionally, activation of AXL in cancer cells induces a cancer stem cell phenotype, regulates self-renewal, and resistance to chemotherapies and receptor tyrosine kinase inhibitors (Asiedu et al., 2014; Byers et al., 2013; Wilson et al., 2014). Future studies will help elucidate the AXL-dependent signaling pathways driving EMT, self-renewal and resistance to therapy.

EMT-induced formation of CSCs

It is becoming increasingly clear that the induction of EMT can generate cancer stem cells (CSCs). CSCs generated through EMT are capable of initiating tumor formation, self-renewing, and contribute to metastases and resistance to conventional therapies (Singh and Settleman, 2010). The first direct link between EMT and gain of epithelial stem cell properties was provided by Mani and colleagues (Mani et al., 2008), who demonstrated that induction of EMT in normal and malignant mammary epithelial cells resulted in the acquisition of stem-cell-like characteristics. Several studies have provided additional evidence that highly aggressive and undifferentiated tumors have both EMT and stem cell characteristics (Ben-Porath et al., 2008; Hennessy et al., 2009; Sarrio et al., 2008; Taube et al., 2010). Mesenchymal cells and CSCs generated from an EMT are highly resistant to therapies (Creighton et al., 2009; Shintani et al., 2011; Thomson et al., 2005). This was highlighted in a study by Creighton et al., who

demonstrated that residual tumor cells surviving after conventional chemotherapy treatment were enriched with EMT and stem cell markers (Creighton et al., 2009). Consistent with this, induction of EMT in cancer cells promotes resistance to chemotherapies and RTK inhibitors (Shintani et al., 2011; Thomson et al., 2005; Thomson et al., 2008). These studies provide evidence that EMT mediates self-renewal of cancer cells and response to chemotherapies and targeted therapies.

Targeting EMT in cancer

Induction of EMT in human cancer cells promotes survival and self-renewal (Mani et al., 2008; Polyak and Weinberg, 2009b) and confers the ability to migrate, survive and self-renew in foreign environments, all essential requirements for metastatic behavior. Several strategies for targeting the EMT pathways are being investigated to control cancer cell invasion, self-renewal, and metastatic dissemination (Davis et al., 2014). Approaches to target EMT involve inhibiting the induction of EMT through targeting extracellular signals as well as targeting critical intracellular signal transduction pathways. The preferred strategy to target the induction of EMT has been through the suppression of RTK signaling using receptor antagonists, antibody inhibitors or small molecule inhibitors. Examples of RTK inhibitors identified to inhibit EMT induction *in vitro* include EGF receptor tyrosine kinase inhibitor AG1487 and TGFβ receptor kinase inhibitor SB431542 (Halder et al., 2005; Lo et al., 2007). Other inhibitors that have been validated to inhibit EMT in cancer cell lines include AXL inhibitor

R428 (Holland et al., 2010), c-Met inhibitor SU11274 (Toiyama et al., 2012), and IGF1R inhibitor AG1024 (Vazquez-Martin et al., 2013). Although these studies provided important preliminary evidence supporting the clinical development of EMT inhibitors, there are limitations to the homogeneous cell culture technique used in these studies as they do not replicate the tissue interactions and numerous microenvironment signals present in normal physiological conditions as cells are undergoing EMT. Complementary *in vivo* approaches are required to validate the efficacy of these inhibitors.

Another valid approach to inhibiting EMT is to target the critical intracellular signaling transduction pathways. Recent advances in the generation of small molecule inhibitors targeting transcription factors (Siddiquee et al., 2007) has provided a potential strategy to develop novel ways of targeting of EMT-associated TFs such as Snail, Slug, Zeb, and Twist to block EMT. The effectiveness of targeting Snail family members to inhibit EMT during both development and cancer progression has already been established (Cano et al., 2000; Carver et al., 2001; Kudo-Saito et al., 2009; Peinado et al., 2004; Vincent et al., 2009), which supports the notion to target EMT TF as a therapeutic strategy.

Another approach currently being explored is targeting the mesenchymal phenotype. Proteins that make up the mesenchymal phenotype like vimentin, matrix metalloprotease enzymes, and fibronectin proteins are currently being investigated as therapeutic targets (Davis et al., 2014). A bioactive compound called Withaferin-A has been shown to inhibit the intermediate filament protein

Vimentin, block cell invasion and migration of cancer cells *in vitro* and suppress metastasis in xenograft tumor models (Lahat et al., 2010; Thaiparambil et al., 2011). Methods to target CSCs generated from an EMT are also being investigated. Monoclonal antibodies targeting CD44, a cell surface marker of CSCs, are currently under development (Marangoni et al., 2009). Additionally, the identification of the potassium ionophore Salinomycin as a selective inhibitor of CSCs generated by EMT induction has demonstrated promising results as it has significant toxicity against CSCs (Gupta et al., 2009). Further studies are required to identify the efficacy of Salinomycin *in vivo* and to determine its effect on normal adult stem cells.

The pharmacological targets identified so far all have implications in cell signaling that go beyond regulating EMT and should be considered carefully to avoid severe toxicity in cancer patients. Furthermore, inhibitors of EMT induction may only be effective as an adjuvant therapy to prevent metastasis and eradicate residual tumor cells and prevent tumor reoccurrence. Treating patients who have pre-existing metastasis with inhibitors of EMT or the mesenchymal phenotype may accelerate epithelialization of disseminated mesenchymal cells or promote the formation of more metastases. Only comprehensive *in vivo* experimental approaches will help determine the most effective inhibitors of EMT, stemness, and therapeutic resistance.

While identifying inhibitors of EMT is of great therapeutic interest there are currently no effective EMT inhibitors in the clinic. This is due to both our incomplete understanding of EMT, and the difficulty in modeling the physiological

complexity of EMT in cell culture systems (Davis et al., 2014). Additionally, complementary *in vivo* approaches to identify effective EMT inhibitors have been limited by the difficulty in labeling and observing EMT in a living animal. In my graduate studies we have overcome this challenge by developing a zebrafish *in vivo* EMT reporter and implemented it in a chemical genetic screen to identify small molecules inhibitors of EMT (Chapter 2). Through our studies we identified a potent small molecule inhibitor of EMT, called TP-0903, that functions by increasing retinoic acid signaling to block EMT during development. Additionally our findings provided insight into candidate signaling pathways, like AXL RTK, that could be tested for promoting cancer cell invasion *in vivo* using an established zebrafish melanoma model (Chapter 3). These studies present the first animal model that can be used to identify effective small molecule inhibitors of EMT *in vivo* and novel signaling pathways regulating cancer progression to help eliminate metastatic disease.

References

Asiedu, M. K., Beauchamp-Perez, F. D., Ingle, J. N., Behrens, M. D., Radisky, D. C. and Knutson, K. L. (2014). AXL induces epithelial-to-mesenchymal transition and regulates the function of breast cancer stem cells. *Oncogene* **33**, 1316-24.

Bakin, A. V., Tomlinson, A. K., Bhowmick, N. A., Moses, H. L. and Arteaga, C. L. (2000). Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* **275**, 36803-10.

Batlle, E., Sancho, E., Franci, C., Dominguez, D., Monfar, M., Baulida, J. and Garcia De Herreros, A. (2000). The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol* **2**, 84-9.

Ben-Porath, I., Thomson, M. W., Carey, V. J., Ge, R., Bell, G. W., Regev, A. and Weinberg, R. A. (2008). An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet* **40**, 499-507.

Bhowmick, N. A., Ghiassi, M., Bakin, A., Aakre, M., Lundquist, C. A., Engel, M. E., Arteaga, C. L. and Moses, H. L. (2001). Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell* **12**, 27-36.

Billottet, C., Tuefferd, M., Gentien, D., Rapinat, A., Thiery, J. P., Broet, P. and Jouanneau, J. (2008). Modulation of several waves of gene expression during FGF-1 induced epithelial-mesenchymal transition of carcinoma cells. *J Cell Biochem* **104**, 826-39.

Byers, L. A., Diao, L., Wang, J., Saintigny, P., Girard, L., Peyton, M., Shen, L., Fan, Y., Giri, U., Tumula, P. K. et al. (2013). An epithelial-mesenchymal transition gene signature predicts resistance to EGFR and PI3K inhibitors and identifies Axl as a therapeutic target for overcoming EGFR inhibitor resistance. *Clin Cancer Res* **19**, 279-90.

Cano, A., Perez-Moreno, M. A., Rodrigo, I., Locascio, A., Blanco, M. J., del Barrio, M. G., Portillo, F. and Nieto, M. A. (2000). The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* **2**, 76-83.

Carver, E. A., Jiang, R., Lan, Y., Oram, K. F. and Gridley, T. (2001). The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. *Mol Cell Biol* **21**, 8184-8.

Creighton, C. J., Li, X., Landis, M., Dixon, J. M., Neumeister, V. M., Sjolund, A., Rimm, D. L., Wong, H., Rodriguez, A., Herschkowitz, J. I. et al. (2009). Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. *Proc Natl Acad Sci U S A* **106**, 13820-5.

Davis, F. M., Stewart, T. A., Thompson, E. W. and Monteith, G. R. (2014). Targeting EMT in cancer: opportunities for pharmacological intervention. *Trends Pharmacol Sci* **35**, 479-88.

Doehn, U., Hauge, C., Frank, S. R., Jensen, C. J., Duda, K., Nielsen, J. V., Cohen, M. S., Johansen, J. V., Winther, B. R., Lund, L. R. et al. (2009). RSK is a principal effector of the RAS-ERK pathway for eliciting a coordinate promotile/invasive gene program and phenotype in epithelial cells. *Mol Cell* **35**, 511-22.

Fidler, I. J. (2003). The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer* **3**, 453-8.

Fraga, M. F., Herranz, M., Espada, J., Ballestar, E., Paz, M. F., Ropero, S., Erkek, E., Bozdogan, O., Peinado, H., Niveleau, A. et al. (2004). A mouse skin multistage carcinogenesis model reflects the aberrant DNA methylation patterns of human tumors. *Cancer Res* **64**, 5527-34.

Fujita, N., Jaye, D. L., Kajita, M., Geigerman, C., Moreno, C. S. and Wade, P. A. (2003). MTA3, a Mi-2/NuRD complex subunit, regulates an invasive growth pathway in breast cancer. *Cell* **113**, 207-19.

Gjerdrum, C., Tiron, C., Hoiby, T., Stefansson, I., Haugen, H., Sandal, T., Collett, K., Li, S., McCormack, E., Gjertsen, B. T. et al. (2010). Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival. *Proc Natl Acad Sci U S A* **107**, 1124-9.

Gregory, P. A., Bert, A. G., Paterson, E. L., Barry, S. C., Tsykin, A., Farshid, G., Vadas, M. A., Khew-Goodall, Y. and Goodall, G. J. (2008). The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* **10**, 593-601.

Grosshans, J. and Wieschaus, E. (2000). A genetic link between morphogenesis and cell division during formation of the ventral furrow in Drosophila. *Cell* **101**, 523-31.

Gupta, P. B., Kuperwasser, C., Brunet, J. P., Ramaswamy, S., Kuo, W. L., Gray, J. W., Naber, S. P. and Weinberg, R. A. (2005). The melanocyte differentiation program predisposes to metastasis after neoplastic transformation. *Nat Genet* **37**, 1047-54.

Gupta, P. B., Onder, T. T., Jiang, G., Tao, K., Kuperwasser, C., Weinberg, R. A. and Lander, E. S. (2009). Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell* **138**, 645-59.

Hafizi, S. and Dahlback, B. (2006). Signalling and functional diversity within the Axl subfamily of receptor tyrosine kinases. *Cytokine Growth Factor Rev* **17**, 295-304.

Halder, S. K., Beauchamp, R. D. and Datta, P. K. (2005). A specific inhibitor of TGF-beta receptor kinase, SB-431542, as a potent antitumor agent for human cancers. *Neoplasia* **7**, 509-21.

Hanahan, D. and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* **144**, 646-74.

Haraguchi, M., Okubo, T., Miyashita, Y., Miyamoto, Y., Hayashi, M., Crotti, T. N., McHugh, K. P. and Ozawa, M. (2008). Snail regulates cell-matrix adhesion by regulation of the expression of integrins and basement membrane proteins. *J Biol Chem* **283**, 23514-23.

Hay, E. D. (1995). An overview of epithelio-mesenchymal transformation. *Acta Anat (Basel)* **154**, 8-20.

Heisenberg, C. P. and Solnica-Krezel, L. (2008). Back and forth between cell fate specification and movement during vertebrate gastrulation. *Curr Opin Genet Dev* **18**, 311-6.

Hennessy, B. T., Gonzalez-Angulo, A. M., Stemke-Hale, K., Gilcrease, M. Z., Krishnamurthy, S., Lee, J. S., Fridlyand, J., Sahin, A., Agarwal, R., Joy, C. et al. (2009). Characterization of a naturally occurring breast cancer subset enriched in epithelial-to-mesenchymal transition and stem cell characteristics. *Cancer Res* **69**, 4116-24.

Herranz, N., Pasini, D., Diaz, V. M., Franci, C., Gutierrez, A., Dave, N., Escriva, M., Hernandez-Munoz, I., Di Croce, L., Helin, K. et al. (2008). Polycomb complex 2 is required for E-cadherin repression by the Snail1 transcription factor. *Mol Cell Biol* **28**, 4772-81.

Hlubek, F., Brabletz, T., Budczies, J., Pfeiffer, S., Jung, A. and Kirchner, T. (2007). Heterogeneous expression of Wnt/beta-catenin target genes within colorectal cancer. *Int J Cancer* **121**, 1941-8.

Hoek, K., Rimm, D. L., Williams, K. R., Zhao, H., Ariyan, S., Lin, A., Kluger, H. M., Berger, A. J., Cheng, E., Trombetta, E. S. et al. (2004). Expression profiling reveals novel pathways in the transformation of melanocytes to melanomas. *Cancer Res* **64**, 5270-82.

Holland, S. J., Pan, A., Franci, C., Hu, Y., Chang, B., Li, W., Duan, M., Torneros, A., Yu, J., Heckrodt, T. J. et al. (2010). R428, a selective small molecule inhibitor of Axl kinase, blocks tumor spread and prolongs survival in models of metastatic breast cancer. *Cancer Res* **70**, 1544-54.

Hoot, K. E., Lighthall, J., Han, G., Lu, S. L., Li, A., Ju, W., Kulesz-Martin, M., Bottinger, E. and Wang, X. J. (2008). Keratinocyte-specific Smad2 ablation results in increased epithelial-mesenchymal transition during skin cancer formation and progression. *J Clin Invest* **118**, 2722-32.

Huang, R. Y., Guilford, P. and Thiery, J. P. (2012). Early events in cell adhesion and polarity during epithelial-mesenchymal transition. *J Cell Sci* **125**, 4417-22.

Jeanes, A., Gottardi, C. J. and Yap, A. S. (2008). Cadherins and cancer: how does cadherin dysfunction promote tumor progression? *Oncogene* **27**, 6920-9.

Jorda, M., Olmeda, D., Vinyals, A., Valero, E., Cubillo, E., Llorens, A., Cano, A. and Fabra, A. (2005). Upregulation of MMP-9 in MDCK epithelial cell line in response to expression of the Snail transcription factor. *J Cell Sci* **118**, 3371-85.

Julien, S., Puig, I., Caretti, E., Bonaventure, J., Nelles, L., van Roy, F., Dargemont, C., de Herreros, A. G., Bellacosa, A. and Larue, L. (2007). Activation of NF-kappaB by Akt upregulates Snail expression and induces epithelium mesenchyme transition. *Oncogene* **26**, 7445-56.

Kaimori, A., Potter, J., Kaimori, J. Y., Wang, C., Mezey, E. and Koteish, A. (2007). Transforming growth factor-beta1 induces an epithelial-to-mesenchymal transition state in mouse hepatocytes in vitro. *J Biol Chem* **282**, 22089-101.

Kalluri, R. and Weinberg, R. A. (2009). The basics of epithelial-mesenchymal transition. *J Clin Invest* **119**, 1420-8.

Kang, Y., Chen, C. R. and Massague, J. (2003). A self-enabling TGFbeta response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. *Mol Cell* **11**, 915-26.

Katsuno, Y., Lamouille, S. and Derynck, R. (2013). TGF-beta signaling and epithelial-mesenchymal transition in cancer progression. *Curr Opin Oncol* **25**, 76-84.

Kolsch, V., Seher, T., Fernandez-Ballester, G. J., Serrano, L. and Leptin, M. (2007). Control of Drosophila gastrulation by apical localization of

adherens junctions and RhoGEF2. *Science* **315**, 384-6.

Kudo-Saito, C., Shirako, H., Takeuchi, T. and Kawakami, Y. (2009). Cancer metastasis is accelerated through immunosuppression during Snail-induced EMT of cancer cells. *Cancer Cell* **15**, 195-206.

Lahat, G., Zhu, Q. S., Huang, K. L., Wang, S., Bolshakov, S., Liu, J., Torres, K., Langley, R. R., Lazar, A. J., Hung, M. C. et al. (2010). Vimentin is a novel anti-cancer therapeutic target; insights from in vitro and in vivo mice xenograft studies. *PLoS One* **5**, e10105.

Lamouille, S., Connolly, E., Smyth, J. W., Akhurst, R. J. and Derynck, R. (2012). TGF-beta-induced activation of mTOR complex 2 drives epithelial-mesenchymal transition and cell invasion. *J Cell Sci* **125**, 1259-73.

Lamouille, S. and Derynck, R. (2007). Cell size and invasion in TGF-beta-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. *J Cell Biol* **178**, 437-51.

Lamouille, S., Xu, J. and Derynck, R. (2014). Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* **15**, 178-96.

Le Douarin, N. and Kalcheim, C. (1999). The neural crest. Cambridge, UK ; New York, NY, USA: Cambridge University Press.

Lee, R. T., Nagai, H., Nakaya, Y., Sheng, G., Trainor, P. A., Weston, J. A. and Thiery, J. P. (2013). Cell delamination in the mesencephalic neural fold and its implication for the origin of ectomesenchyme. *Development* **140**, 4890-902.

Lim, J. and Thiery, J. P. (2012). Epithelial-mesenchymal transitions: insights from development. *Development* **139**, 3471-86.

Linger, R. M., Keating, A. K., Earp, H. S. and Graham, D. K. (2008). TAM receptor tyrosine kinases: biologic functions, signaling, and potential therapeutic targeting in human cancer. *Adv Cancer Res* **100**, 35-83.

Linger, R. M., Keating, A. K., Earp, H. S. and Graham, D. K. (2010). Taking aim at Mer and Axl receptor tyrosine kinases as novel therapeutic targets in solid tumors. *Expert Opin Ther Targets* **14**, 1073-90.

Liu, Y. N., Yin, J. J., Abou-Kheir, W., Hynes, P. G., Casey, O. M., Fang, L., Yi, M., Stephens, R. M., Seng, V., Sheppard-Tillman, H. et al. (2013). MiR-1 and miR-200 inhibit EMT via Slug-dependent and tumorigenesis via Slug-independent mechanisms. *Oncogene* **32**, 296-306.

Lo, H. W., Hsu, S. C., Xia, W., Cao, X., Shih, J. Y., Wei, Y., Abbruzzese, J. L., Hortobagyi, G. N. and Hung, M. C. (2007). Epidermal growth factor receptor cooperates with signal transducer and activator of transcription 3 to induce epithelial-mesenchymal transition in cancer cells via up-regulation of TWIST gene expression. *Cancer Res* **67**, 9066-76.

Lu, Z., Ghosh, S., Wang, Z. and Hunter, T. (2003). Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion. *Cancer Cell* **4**, 499-515.

Ma, L., Young, J., Prabhala, H., Pan, E., Mestdagh, P., Muth, D., Teruya-Feldstein, J., Reinhardt, F., Onder, T. T., Valastyan, S. et al. (2010). miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. *Nat Cell Biol* **12**, 247-56.

Makrodouli, E., Oikonomou, E., Koc, M., Andera, L., Sasazuki, T., Shirasawa, S. and Pintzas, A. (2011). BRAF and RAS oncogenes regulate Rho GTPase pathways to mediate migration and invasion properties in human colon cancer cells: a comparative study. *Mol Cancer* **10**, 118.

Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., Brooks, M., Reinhard, F., Zhang, C. C., Shipitsin, M. et al. (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133**, 704-15.

Marangoni, E., Lecomte, N., Durand, L., de Pinieux, G., Decaudin, D., Chomienne, C., Smadja-Joffe, F. and Poupon, M. F. (2009). CD44 targeting reduces tumour growth and prevents post-chemotherapy relapse of human breast cancer xenografts. *Br J Cancer* **100**, 918-22.

Marchetti, A., Colletti, M., Cozzolino, A. M., Steindler, C., Lunadei, M., Mancone, C. and Tripodi, M. (2008). ERK5/MAPK is activated by TGFbeta in hepatocytes and required for the GSK-3beta-mediated Snail protein stabilization. *Cell Signal* **20**, 2113-8.

Moustakas, A. and Heldin, C. H. (2005). Non-Smad TGF-beta signals. *J Cell Sci* **118**, 3573-84.

Murray, S. A. and Gridley, T. (2006). Snail family genes are required for left-right asymmetry determination, but not neural crest formation, in mice. *Proc Natl Acad Sci U S A* **103**, 10300-4.

Nawshad, A., Medici, D., Liu, C. C. and Hay, E. D. (2007). TGFbeta3 inhibits E-cadherin gene expression in palate medial-edge epithelial cells through a Smad2-Smad4-LEF1 transcription complex. *J Cell Sci* **120**, 1646-53.

Nieto, M. A. (2011). The ins and outs of the epithelial to mesenchymal transition in health and disease. *Annu Rev Cell Dev Biol* **27**, 347-76.

Nieto, M. A., Sargent, M. G., Wilkinson, D. G. and Cooke, J. (1994). Control of cell behavior during vertebrate development by Slug, a zinc finger gene. *Science* **264**, 835-9.

Ozdamar, B., Bose, R., Barrios-Rodiles, M., Wang, H. R., Zhang, Y. and Wrana, J. L. (2005). Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. *Science* **307**, 1603-9.

Peinado, H., Ballestar, E., Esteller, M. and Cano, A. (2004). Snail mediates E-cadherin repression by the recruitment of the Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex. *Mol Cell Biol* **24**, 306-19.

Polyak, K. and Weinberg, R. A. (2009). Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer* **9**, 265-73.

Portella, G., Cumming, S. A., Liddell, J., Cui, W., Ireland, H., Akhurst, R. J. and Balmain, A. (1998). Transforming growth factor beta is essential for spindle cell conversion of mouse skin carcinoma in vivo: implications for tumor invasion. *Cell Growth Differ* **9**, 393-404.

Powell, D. R., Blasky, A. J., Britt, S. G. and Artinger, K. B. (2013). Riding the crest of the wave: parallels between the neural crest and cancer in epithelial-to-mesenchymal transition and migration. *Wiley Interdiscip Rev Syst Biol Med* **5**, 511-22.

Puisieux, A., Brabletz, T. and Caramel, J. (2014). Oncogenic roles of EMT-inducing transcription factors. *Nat Cell Biol* **16**, 488-94.

Ridley, A. J. (2011). Life at the leading edge. *Cell* **145**, 1012-22.

Sarrio, D., Rodriguez-Pinilla, S. M., Hardisson, D., Cano, A., Moreno-Bueno, G. and Palacios, J. (2008). Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. *Cancer Res* **68**, 989-97.

Savagner, P., Yamada, K. M. and Thiery, J. P. (1997). The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition. *J Cell Biol* **137**, 1403-19.

Shapiro, I. M., Cheng, A. W., Flytzanis, N. C., Balsamo, M., Condeelis, J. S., Oktay, M. H., Burge, C. B. and Gertler, F. B. (2011). An EMT-driven alternative splicing program occurs in human breast cancer and modulates cellular phenotype. *PLoS Genet* **7**, e1002218.

Shen, X., Li, J., Hu, P. P., Waddell, D., Zhang, J. and Wang, X. F. (2001). The activity of guanine exchange factor NET1 is essential for transforming growth factor-beta-mediated stress fiber formation. *J Biol Chem* **276**, 15362-8.

Shintani, Y., Okimura, A., Sato, K., Nakagiri, T., Kadota, Y., Inoue, M., Sawabata, N., Minami, M., Ikeda, N., Kawahara, K. et al. (2011). Epithelial to mesenchymal transition is a determinant of sensitivity to chemoradiotherapy in non-small cell lung cancer. *Ann Thorac Surg* **92**, 1794-804; discussion 1804.

Shirakihara, T., Horiguchi, K., Miyazawa, K., Ehata, S., Shibata, T., Morita, I., Miyazono, K. and Saitoh, M. (2011). TGF-beta regulates isoform switching of FGF receptors and epithelial-mesenchymal transition. *EMBO J* **30**, 783-95.

Shirakihara, T., Saitoh, M. and Miyazono, K. (2007). Differential regulation of epithelial and mesenchymal markers by deltaEF1 proteins in epithelial mesenchymal transition induced by TGF-beta. *Mol Biol Cell* **18**, 3533-44.

Siddiquee, K., Zhang, S., Guida, W. C., Blaskovich, M. A., Greedy, B., Lawrence, H. R., Yip, M. L., Jove, R., McLaughlin, M. M., Lawrence, N. J. et al. (2007). Selective chemical probe inhibitor of Stat3, identified through structure-based virtual screening, induces antitumor activity. *Proc Natl Acad Sci U S A* **104**, 7391-6.

Singh, A. and Settleman, J. (2010). EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* **29**, 4741-51.

Tang, S., Snider, P., Firulli, A. B. and Conway, S. J. (2010). Trigenic neural crest-restricted Smad7 over-expression results in congenital craniofacial and cardiovascular defects. *Dev Biol* **344**, 233-47.

Taube, J. H., Herschkowitz, J. I., Komurov, K., Zhou, A. Y., Gupta, S., Yang, J., Hartwell, K., Onder, T. T., Gupta, P. B., Evans, K. W. et al. (2010). Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. *Proc Natl Acad Sci U S A* **107**, 15449-54.

Thaiparambil, J. T., Bender, L., Ganesh, T., Kline, E., Patel, P., Liu, Y., Tighiouart, M., Vertino, P. M., Harvey, R. D., Garcia, A. et al. (2011). Withaferin A inhibits breast cancer invasion and metastasis at sub-cytotoxic doses by inducing vimentin disassembly and serine 56 phosphorylation. *Int J Cancer* **129**, 2744-55.

Theveneau, E. and Mayor, R. (2012). Neural crest delamination and

migration: from epithelium-to-mesenchyme transition to collective cell migration. *Dev Biol* **366**, 34-54.

Thiery, J. P., Acloque, H., Huang, R. Y. and Nieto, M. A. (2009). Epithelial-mesenchymal transitions in development and disease. *Cell* **139**, 871-90.

Thiery, J. P. and Sleeman, J. P. (2006). Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* **7**, 131-42.

Thomson, S., Buck, E., Petti, F., Griffin, G., Brown, E., Ramnarine, N., Iwata, K. K., Gibson, N. and Haley, J. D. (2005). Epithelial to mesenchymal transition is a determinant of sensitivity of non-small-cell lung carcinoma cell lines and xenografts to epidermal growth factor receptor inhibition. *Cancer Res* **65**, 9455-62.

Thomson, S., Petti, F., Sujka-Kwok, I., Epstein, D. and Haley, J. D. (2008). Kinase switching in mesenchymal-like non-small cell lung cancer lines contributes to EGFR inhibitor resistance through pathway redundancy. *Clin Exp Metastasis* **25**, 843-54.

Toiyama, Y., Yasuda, H., Saigusa, S., Matushita, K., Fujikawa, H., Tanaka, K., Mohri, Y., Inoue, Y., Goel, A. and Kusunoki, M. (2012). Co-expression of hepatocyte growth factor and c-Met predicts peritoneal dissemination established by autocrine hepatocyte growth factor/c-Met signaling in gastric cancer. *Int J Cancer* **130**, 2912-21.

Trelstad, R. L., Hay, E. D. and Revel, J. D. (1967). Cell contact during early morphogenesis in the chick embryo. *Dev Biol* **16**, 78-106.

Valles, A. M., Boyer, B., Tarone, G. and Thiery, J. P. (1996). Alpha 2 beta 1 integrin is required for the collagen and FGF-1 induced cell dispersion in a rat bladder carcinoma cell line. *Cell Adhes Commun* **4**, 187-99.

Vardouli, L., Moustakas, A. and Stournaras, C. (2005). LIM-kinase 2 and cofilin phosphorylation mediate actin cytoskeleton reorganization induced by transforming growth factor-beta. *J Biol Chem* **280**, 11448-57.

Vazquez-Martin, A., Cufi, S., Oliveras-Ferraros, C., Torres-Garcia, V. Z., Corominas-Faja, B., Cuyas, E., Bonavia, R., Visa, J., Martin-Castillo, B., Barrajon-Catalan, E. et al. (2013). IGF-1R/epithelial-to-mesenchymal transition (EMT) crosstalk suppresses the erlotinib-sensitizing effect of EGFR exon 19 deletion mutations. *Sci Rep* **3**, 2560.

Vincent, T., Neve, E. P., Johnson, J. R., Kukalev, A., Rojo, F., Albanell, J., Pietras, K., Virtanen, I., Philipson, L., Leopold, P. L. et al.

(2009). A SNAIL1-SMAD3/4 transcriptional repressor complex promotes TGF-beta mediated epithelial-mesenchymal transition. *Nat Cell Biol* **11**, 943-50.

Warzecha, C. C., Jiang, P., Amirikian, K., Dittmar, K. A., Lu, H., Shen, S., Guo, W., Xing, Y. and Carstens, R. P. (2010). An ESRP-regulated splicing programme is abrogated during the epithelial-mesenchymal transition. *EMBO J* **29**, 3286-300.

Weigelt, B., Peterse, J. L. and van 't Veer, L. J. (2005). Breast cancer metastasis: markers and models. *Nat Rev Cancer* **5**, 591-602.

Wilson, C., Ye, X., Pham, T., Lin, E., Chan, S., McNamara, E., Neve, R. M., Belmont, L., Koeppen, H., Yauch, R. L. et al. (2014). AXL inhibition sensitizes mesenchymal cancer cells to antimitotic drugs. *Cancer Res* **74**, 5878-90.

Wu, S. Y., Ferkowicz, M. and McClay, D. R. (2007). Ingression of primary mesenchyme cells of the sea urchin embryo: a precisely timed epithelial mesenchymal transition. *Birth Defects Res C Embryo Today* **81**, 241-52.

Wu, S. Y., Yang, Y. P. and McClay, D. R. (2008). Twist is an essential regulator of the skeletogenic gene regulatory network in the sea urchin embryo. *Dev Biol* **319**, 406-15.

Yang, J., Mani, S. A., Donaher, J. L., Ramaswamy, S., Itzykson, R. A., Come, C., Savagner, P., Gitelman, I., Richardson, A. and Weinberg, R. A. (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* **117**, 927-39.

Yang, L., Lin, C. and Liu, Z. R. (2006). P68 RNA helicase mediates PDGF-induced epithelial mesenchymal transition by displacing Axin from beta-catenin. *Cell* **127**, 139-55.

CHAPTER 2

NOVEL EMT REPORTER FOR RAPID IN VIVO DRUG SCREENING

This research is under review for publication in Disease Models & Mechanisms. Laura Jimenez, Jindong Wang, David Bearss, Cicely A. Jette, Rodney A. Stewart. Novel EMT Reporter for Rapid In Vivo Drug Screening.

Abstract

The epithelial to mesenchymal transition (EMT) is a highly conserved morphogenetic program essential for embryogenesis, regeneration and cancer metastasis. In cancer cells, EMT also triggers cellular reprogramming and resistance to chemotherapy, which underlie disease relapse and decreased survival. Hence, identifying compounds that block EMT is essential to prevent or eradicate disseminated tumor cells. Here, we establish a novel *in vivo* EMT reporter in zebrafish for rapid drug screening called *Tg(snai1b:GFP)*, which labels Snail1-expressing cells undergoing EMT during development, including dorsal neural tube progenitors that form neural crest. Treating *Tg(snai1b:GFP)* embryos with small molecule compounds identified a multi-kinase inhibitor TP-0903, which inhibited neuroepithelial EMT. RNA-Seq analysis and chemical rescue experiments show TP-0903 acts through activation of retinoic acid (RA) biosynthesis and signaling. These studies identify TP-0903 as a new therapeutic for activating RA *in vivo* and indicate the previous enigmatic success of RA in eliminating disseminated cancer cells is through inhibition of EMT.

Resource impact

Induction of the Epithelial to Mesenchymal Transition (EMT) in cancer cells promotes acquisition of invasive cellular morphologies, stem-cell-like properties and prosurvival mechanisms that contribute to therapeutic resistance, relapse and decreased overall survival. Identifying EMT inhibitors for clinical use is of great therapeutic interest as adjuvant therapies to eliminate rare or dormant

cells, but no effective EMT small molecule compounds have yet entered the clinic. This is due to an incomplete understanding of the molecular mechanisms regulating EMT in both normal development and disease. In addition, a number of compounds that inhibit EMT *in vitro* fail to inhibit EMT *in vivo*, due to the fact that 1) cell culture assays do not mimic the complexity of the normal physiological or disease environments and 2) multiple compensatory pathways likely function in parallel to induce EMT *in vivo*. Clearly complementary *in vivo* approaches are needed to screen and identify effective EMT inhibitors. Unfortunately, difficulty in labeling and observing EMT in living animals in large numbers for screening has prevented us from using such approaches.

In this study, we have overcome this roadblock and developed a zebrafish *in vivo* EMT reporter to rapidly screen small molecules for their ability to inhibit EMT. We identified a novel small molecule EMT inhibitor, called TP-0903, that potently inhibits EMT *in vivo*, and determined its mechanism of action in this process through promoting retinoic acid (RA) biosynthesis. We also show for the first time that RA itself inhibits EMT (despite numerous studies showing RA affects other aspects of neural development). These results may explain the previous enigmatic ability of RA as a successful adjuvant therapy to eradicate minimal residue disease and improve cancer patient survival. We also discuss the potential future use of TP-0903 as a new adjuvant therapy for metastatic disease.

Introduction

The conversion of epithelial cells into migratory, invasive mesenchymal cells is a fundamental morphogenetic process during development and regeneration. Induction of epithelial-mesenchymal transition (EMT) causes epithelial cells to lose intracellular junctions, apical-basal polarity and gain cytoskeleton reorganization that is prerequisite for motility and invasion through surrounding tissue (Hay, 1995). In addition, several studies have demonstrated that reactivation of developmental EMT programs in cancer cells constitutes a key step during metastasis (Thiery et al., 2009). EMT can award cancer cells with invasive properties to allow dissemination from the primary tumor and promote the acquisition of stem cell like properties (Mani et al., 2008), therapeutic resistance (Kurrey et al., 2009; Li et al., 2009), increased survival and immune-suppression (Polyak and Weinberg, 2009), all of which contribute to poor patient prognosis. For these reasons targeting EMT in cancer patients has gained significant therapeutic interest.

Our understanding of the cellular and molecular pathways controlling EMT in normal or cancerous cells remains incomplete, hindering efforts to rationally target EMT in the clinic. Indeed, most current small molecule EMT inhibitors were discovered through unbiased cell-based *in vitro* screening techniques (Davis et al., 2014). However, these assays are usually restricted to single homogeneous cell types and do not fully recapitulate the complex physiological environment in which other cell types and different extracellular matrix (ECM) components impact EMT induction. Establishing *in vivo* EMT reporter assays for rapid

screening is essential to complement conventional cell-based assays to identify the most effective EMT inhibitors for human disease. In addition, whole animal–based EMT reporter models allow direct assessment of compounds on normal cell populations to determine tissue-specific toxicities, as well as discovery of novel molecular pathways controlling physiological EMT that can be rationally targeted.

The embryonic dorsal neural tube of vertebrates is an excellent system to identify mechanisms controlling EMT because these cells undergo highly predictable EMT movements to form migratory neural crest (NC) cells that then migrate collectively or individually to generate a variety of cell types, such as cardiac, craniofacial, and pigment cells, as well as neurons and glia of the peripheral nervous system (Green et al., 2015). Thus, defects in dorsal neural tube EMT and NC migration underlie a number of human congenital diseases, particularly craniofacial abnormalities. NC-derived lineages are also the origin of some of the most highly metastatic human cancers, such as melanoma and neuroblastoma, suggesting these cancers can readily reprogram their embryonic EMT and cell migration mechanisms to promote rapid tumor dissemination. Indeed, seminal work that originally linked EMT to cancer metastasis showed genes expressed during neural tube EMT are aberrantly activated during metastasis (Gupta and Massague, 2006; Kang and Massague, 2004; Yang et al., 2004). Among these are members of the *Snail* and *Twist* family of transcription factor genes, which repress the expression of epithelial cell adhesion molecules, including E-cadherin, to promote EMT during both development and in metastatic

tumors (Gupta et al., 2005). This suggests that inhibitors of conserved signaling pathways controlling dorsal neural tube EMT will also be excellent therapies for blocking EMT during tumor invasion and/or metastasis.

Based on numerous studies in different vertebrate species, current models suggest that EMT in the dorsal neuroepithelium is induced by the combined actions of a number of growth factors secreted from the epiblast (BMP antagonists), underlying paraxial mesoderm (FGF) and ectoderm (Wnt). These pathways converge at the epithelial neural folds to induce the expression of canonical EMT transcription factors, such as Snail1/2, Twist1 and Zeb1/2, as well as NC "specifier" genes Sox9/10, Foxd3 and tfAP2α (Knecht and Bronner-Fraser, 2002; Sauka-Spengler and Bronner-Fraser, 2008). Thus, a gene regulatory network, mediated primarily through TGFb/BMP and Wnt signaling, is proposed to control Snail1/2 and Twist1 expression and/or stability, which in turn promotes EMT to produce NC cells expressing NC specifier genes. However, there is no direct evidence that any of the above growth factors directly control EMT. Indeed, to our knowledge, there are no examples in which a single pathway can inhibit EMT within the neural tube to cause neuroepithelial progenitors to remain trapped within the neural tube. This suggests multiple compensatory mechanisms control NC EMT at the level of extracellular signaling, transcription factor expression/regulation or both. Identifying the essential, non-redundant signaling pathways required for EMT *in vivo*, or combinations, will be essential to understand normal NC development and allow us to rationally target and inhibit EMT during cancer progression.

Here we develop a novel zebrafish EMT reporter called *Tg(snai1b:GFP)* that fluorescently labels dorsal neural tube progenitors undergoing EMT. In contrast to previous NC reporters in zebrafish, the *Tg(snai1b:GFP)* reporter labels the earliest progenitor population of dorsal neural tube cells *before* NC specifier genes are expressed and without the need for sophisticated confocal imaging. In addition, thousands of *Tg(snai1b:GFP)* embryos can be generated per day by simple genetic crosses for rapid screening, without the need to manually inject DNA constructs into every embryo (Berndt et al., 2008). Indeed, by combining the *Tg(snai1b:GFP)* neuroepithelial lineage marker with the previously established NC reporter, *Tg(sox10:RFP),* we show only *Tg(snai1b:GFP)* labels delaminating dorsal neural tube cells and directly observe the birth of Sox10-positive NC cells from delaminating dorsal neural tube progenitors. We also demonstrate that *Tg(snai1b:GFP)* is readily amenable to chemical genetic screening and identified a novel multi-kinase inhibitor, called TP-0903 that potently blocks EMT and cell migration. Genomic analysis and chemical rescue experiments showed TP-0903 activates RA signaling by increasing biosynthesis. Our findings reveal unexpected and previously unrecognized roles for RA in inhibiting EMT during NC development, and suggest activation of RA signaling through TP-0903 represents a new therapeutic strategy to inhibit EMT and eliminate residual metastatic disease.

Results

Generation of the EMT reporter line: Tg(snai1b:GFP)

Snail transcription factors are inducers of EMT in a number of cell populations during metazoan development (Nieto, 2002). The *snai1b* gene is expressed during early gastrulation in nascent mesodermal cells, in neuroepithelial cells within the neural keel, at the neural plate border and in pre- and postmigratory NC (Figure 2.1A). To directly visualize dorsal neural tube EMT behaviors we isolated and subcloned approximately 3-kb of the *snai1b* promoter into a modified pEGFP-1 plasmid and generated a transgenic strain called *Tg(snai1b:GFP)* (see Methods). Examination of GFP expression in other tissues showed *Tg(snai1b:GFP)* also labels cells during gastrulation and segmentation stages, such as the involuting mesendoderm and somites (Figure 2.2), which express endogenous *snai1b* and undergo EMT, but were not examined further in this study.

To verify the *Tg(snai1b:GFP)* line recapitulates endogenous *snail1b* mRNA expression, we compared the fluorescent GFP pattern in *Tg(snai1b:GFP)* embryos to *snail1b* mRNA *in situ* hybridization at various development stages (Figure 2.1A). At 15 hours postfertilization (hpf), *Tg(snai1b:GFP)* embryos express GFP in neuroepithelial and neural plate border cells, consistent with the expression pattern of endogenous *snail1b* mRNA (Figures 2.1A and Movie S1). At 16.5 hpf, when all cranial NC streams have begun to migrate, GFP-positive cells appear in NC streams. This trend continues at 18 hpf, with GFP expression most prominent in the first and second cranial NC streams and most anterior

neuroepithelium, while there is comparatively less GFP expression in the vagal NC stream, similar to the endogenous *snai1b* mRNA pattern. At 24 hpf, GFP expression is diminished in most cranial NC cells and instead is expressed in delaminating trunk cells (Figure 2.1B) and other cells types (Figure 2.2).

Previous studies demonstrate the *sox10* promoter labels NC cells undergoing delamination behaviors (Berndt et al., 2008). To determine if *Tg(snai1b:GFP)* embryos express GFP in the same neuroepithelial cells as *sox10,* we generated double transgenic *Tg(snai1b:GFP); Tg(sox10:RFP)* animals and analyzed EMT by two color confocal time-lapse imaging during cranial NC development (Movie S2). Unexpectedly, we found only *Tg(snai1b:GFP)* readily labeled dorsal neuroepithelial cells from 15- to 18-hpf (Figure 2.1A), while *Tg(sox10:RFP)* more robustly labeled premigratory NC at the lateral neural plate border and migrating NC cells (Figure 2.1A), with some overlap between the two transgenes in this population. Thus, the *Tg(snai1b:GFP)* transgenic line allows direct visualization of all neuroepithelial progenitors within the neural tube that are predicted to undergo EMT to form NC, while *sox10*-driven transgenes preferentially labels pre- and postmigrating NC.

Visualization of EMT in Tg(snai1b:GFP) animals

Our comparative study of *snai1b*- versus *sox10*-promoter driven expression suggest the *snai1b* promoter readily labels dorsal neuroepithelial cells with potential to undergo EMT (and not the entire neuroepithelium). To confirm this, we analyzed transverse sections through the hindbrain region of double

transgenic *Tg(snai1b:GFP); Tg(sox10:RFP)* animals at different developmental stages (Figure 2.3A). During the neural rod stage (15 hpf), GFP is expressed in distinct two locations, the dorsal half of the neural rod and cells adjacent to the neural plate border, while *sox10*-driven mRFP is expressed in cells adjacent to the neural plate border (Figure 2.3A). During the neural tube stage (18 hpf), GFP expression was further restricted to the dorsal most region of the neural tube and is gradually diminished once cells emerge from the neural tube, labeling only a subset of *sox10*-positive migratory NC cells. In contrast, *sox10*-driven RFP was expressed in pre- and postmigratory NC at 18 hpf, but still absent from the dorsal neural tube (Figure 2.3A).

The dorsal and transverse views of *Tg(snai1b:GFP)* animals suggest the GFP-positive cells within the dorsal neural tube are fated to undergo an EMT and become NC cells. To confirm this, we first analyzed the expression of E-cadherin protein in transverse views of *Tg(snai1b:GFP)* animals, whose loss is a hallmark of EMT and show that dorsally-restricted GFP-positive cells express reduced levels of E-cadherin compared to the rest of the neural tube (Figure 2.3B). We next analyzed time-lapse recordings of double transgenic *Tg(snai1b:GFP); Tg(sox10:RFP)* animals to follow individual cell behaviors as they emerge from the neural tube (Figure 2.3C and Movie S3). Confocal time-lapse analysis confirmed that GFP-positive neuroepithelial cells in *Tg(snai1b:GFP)* animals exhibit stereotypical EMT behaviors (Figure 2.3C). Subsets of neuroepithelial cells contact the apical midline and basal surface. As cells initiate EMT they detach from the apical midline by down-regulating adherence junction and retract

their apical tail. Subsequently, epithelial cells round up near the basal surface and use blebbing motility to translocate out of the neural tube, ultimately gaining directed motility away form the neural tube (Figure 2.3C schematic). These cellular behaviors are consistent with previous live imaging studies of neuroepithelial cells undergoing EMT (Berndt et al., 2008; Clay and Halloran, 2013). Confocal time-lapse analysis of the dorsal midline in *Tg(snai1b:GFP); Tg(sox10:RFP)* animals revealed novel cellular behaviors of the dorsal-most neural tube cells at later stages (Figure 2.4 and Movie S4), which span across the apical midline to contact both sides of the neuroepithelium. During delamination, these cells retract from both sides of the neuroepithelium simultaneously and lose polarity to become rounded. Subsequently, these cells flatten and extend cellular protrusions, such as filopodia and blebs, and begin to express the NC marker *sox10:RFP* (Figure 2.4). These studies show that the *Tg(snai1b:GFP)* line labels multiple delaminating populations of neuroepithelial cells that become NC, and represents a novel whole-animal EMT reporter line that can be used for chemical screening.

Chemical screening approach to identify EMT Inhibitors in vivo

Genetic crosses of the *Tg(snai1b:GFP)* line generate thousands of embryos per day, allowing rapid and direct visualization of EMT behaviors *in vivo* after genetic or chemical perturbations. To determine if the *Tg(snai1b:GFP)* line could be used to identify compounds that block NC EMT *in vivo*, we performed a

chemical screen. *Tg(snai1b:GFP)* embryos were treated with different doses of kinase inhibitors (ranging from 0.1 to 100mM) at the 3- to 8-somite stage (~13 hpf), a time point that 1) avoids developmental delays due to interfering with gastrulation movements and 2) allows growth factor induction to occur at the neural plate border to induce EMT factors, but precedes the onset of most cranial neural tube EMT. Embryos were treated for 6-12 hours (hrs) and then visualized for GFP fluorescence in the neural tube (see Methods and Figure 2.5). Failure of cells to undergo EMT and migrate out of the neuroepithelium was indicated by an increased number of GFP-positive cells within the dorsal neural tube. Surprisingly, under our assay conditions, most chemical compounds did not cause overt EMT phenotypes *in vivo* (Table 2.1), including targeting common kinase and cytoskeletal remodeling pathways, such as the Mapk/Erk, Pi3k/Akt and Rho GTPase pathways, despite these pathway inhibitors having inhibitory EMT effects on individual cells *in vitro* (Berndt et al., 2008; Irie et al., 2005; Zheng et al., 2013). These results suggest that multiple signaling pathways may compensate for each other to drive the EMT program during NC development (see Discussion).

Compounds that primarily target one major signaling pathway did not show a significant effect on NC EMT. However, a multi-kinase inhibitor called TP-0903 (formerly known as HCI-2084) (Mollard et al., 2011) dramatically blocked EMT and NC cell migration (Figure 2.6). TP-0903 was rationally designed to target AXL RTK (IC_{50} = 27 nmol/L), a key oncogenic target that promotes EMT and metastasis (Gjerdrum et al., 2010). TP-0903 also displays strong activity

against 11 other kinases including Aurora A, *Jak2*, *Alk*, and *Abl* and is currently in preclinical development (Tolero Pharmaceuticals, 2015). Confocal time-lapse analysis of *Tg(snai1b:GFP)* embryos treated with TP-0903 between 11- to 19-hpf showed significant accumulation of GFP-positive cells within the developing neuroepithelium (Figure 2.6A, compare Movie S5 and Movie S6). In addition, TP-0903 treatment of *Tg(snai1b:GFP)* embryos halted migration of NC cells that had already exited the neural tube and fused the first two cranial NC streams together (Figure 2.6A). Analysis of developmental timing using the lens placode or other NC-derived tissues (such as pigment) showed that these tissues developed normally, suggesting TP-0903 phenotypes did not cause a general developmental arrest (data not shown, also see Figure 2.8).

To confirm TP-0903 treatment inhibited EMT we analyzed cross sections of *Tg(snai1b:GFP); Tg(sox10:RFP)* embryos treated with TP-0903 and DMSO. In DMSO treated embryos, the majority of the GFP-positive cells have delaminated from the neural tube by 24 hpf to form *sox10*-positive NC. In contrast, TP-0903-treated embryos retained GFP-positive progenitor cells in the dorsal neural tube (Figure 2.6B). Analysis of pan-Cadherin expression in the TP-0903-treated embryos showed that GFP-positive cells expressed elevated Cadherin levels compared to DMSO-treated controls (Figure 2.6C). Finally, to determine if TP-0903 directly affects the expression of canonical EMT transcription factors we analyzed expression of the Snail, Twist and Zeb by RNA *in situ* hybridization (Figure 2.6D). We found TP-0903 caused a complete loss of *twist1a* expression in cranial NC at 18 hpf, while the expression of other EMT transcription factors

was not significantly affected (Figure 2.6D and data not shown). Thus, the *Tg(snai1b:GFP)* transgenic strain is an effective whole-animal based model for identifying novel EMT and cell migration inhibitors, and identifies TP-0903 as a potent EMT inhibitor *in vivo*.

TP-0903 induces a rapid retinoic acid transcriptional response

The loss of EMT transcription factor expression, such as *twist1a*, suggested TP-0903 acts through transcriptional regulation. Therefore, we performed RNA-Seq analysis on control and TP-0903 treated embryos at different time points to determine if TP-0903 elicits an immediate or delayed transcriptional response. We treated zebrafish embryos with TP-0903 or DMSO at the 8-somite stage (13 hpf) when the brain primordium has thickened into the neural keel and cranial neuroepithelial cells are actively undergoing EMT. Embryos were treated for 1-, 4- or 8-hrs and subsequently divided into two groups to 1) extract RNA for gene expression analysis or 2) fixed for *in situ* hybridization to confirm differential expression of candidate genes identified from RNA-Seq in TP-0903 treated embryos (Figure 2.7). Analysis of the relative expression levels of transcripts in TP-0903- and DMSO-treated embryos at different time-points showed TP-0903 induces a rapid transcriptional response in embryos after just 1 hr, which becomes more robust by 4-hrs post-treatment. Analysis of differentially expressed transcripts at 1- and 4-hrs identified several retinoic acid (RA)-target genes, including genes from within the retinoid pathway itself, such as *rxrga, dhrs3a/3b*, and *cyp26a1* and members of the Hox gene

family that harbor retinoic acid response elements (RARE), including *hoxb1a*, *hoxb5b* and *hoxa4a* (Figure 2.7A). Ingenuity Pathway Analysis confirmed that TP-0903 activates an RA transcriptional response in embryos (Figure 2.7B). To validate our findings we examined the expression of two differentially expressed genes, *cyp26a1* and *hoxb1a,* by whole mount *in situ* hybridization in TP-0903 treated embryos (Figure 2.7C). Both *cyp26a1* and *hoxb1a* are direct transcriptional targets of RA and *cyp26a1* is commonly used to report RA activity. Our *in situ* hybridization analysis confirmed the RNA-Seq results and showed that the intensity of *cyp26a1 and hoxb1a* expression was increased in TP-0903 treated embryos compared to controls at 16 hpf (Figure 2.7C). We also treated embryos with RA at the 8-somite stage (13 hpf) and found it mimicked TP-0903 treatment with respect to increased *cyp26a1* and *hoxb1a* expression, although with a much more elevated response (Figure 2.7D).

TP-0903 treatment induces an RA-like response, so we next analyzed the expression pattern of NC genes previously shown to be affected by RA treatment (Ellies et al., 1997; Lee et al., 1995; Matt et al., 2003). We analyzed the expression of *dlx2a* in TP-0903 treated embryos and found that it was inhibited, showing TP-0903, like RA itself, blocks ectomesenchymal differentiation of NC cells (Figure 2.8A). To determine the fate of these cells, we performed *in situ* hybridization analysis with the pan-NC marker *crestin*, which showed the stalled cranial NC cells in TP-0903 embryos were present but fused together, as observed in the *sox10-RFP* analysis (Figure 2.8B). To determine the fate of these cells, we analyzed NC lineage markers and found they differentiate into

mitfa-positive melanocyte precursors (Figure 2.8C). Consistent with this, analysis of mature melanophores at 48 hpf showed that TP-0903 caused the accumulation of pigment cells along the dorsal neural tube (Figure 2.8D). Together, these results show that TP-0903 causes a specific and rapid increase in RA-dependent transcription during NC development, which in turn inhibits both EMT and cell migration, and causes NC cells to adopt a nonectomesenchymal fate at the expense of ectomesenchymal cells.

To directly test if TP-0903 acts through RA signaling we inhibited RA synthesis in TP-0903 treated embryos by co-treating *Tg(snai1b:GFP); Tg(sox10:RFP)* embryos with TP-0903 and Diethylaminobenzaldehyde (DEAB), a potent inhibitor of retinaldehyde dehydrogenase (RALDH), the rate-limiting enzyme required for the final conversion of retinal to RA. Inhibition of RA synthesis by DEAB in TP-0903 treated embryos rescues the NC EMT and cell migration defects (Figure 2.9A and compare Movies S2, S7 and S8). Co-treatment of TP-0903 and DEAB also rescues *twist1a* and *dlx2a* expression and pigment differentiation defects caused by TP-0903 treatment (Figure 2.9C and 2.9D). As expected, DEAB did not rescue RA treated embryos because exogenous RA treatment bypasses the need for Raldh2 and retinal conversion (Figure 2.9A and Movie S9 9). These results confirm that the multi-kinase inhibitor TP-0903 is a novel compound that can elevate RA biosynthesis *in vivo.*

Retinoic acid controls cranial NC EMT, cell migration, and ectomesenchymal differentiation

The genomic, molecular and cellular analysis of TP-0903 treated embryos shows potential new roles for RA signaling in dorsal neural tube EMT. To directly test if RA itself could inhibit EMT, we treated 3-somite stage (11.5 hpf) *Tg(snai1b:GFP); Tg(sox10:RFP)* embryos with RA and found it causes an accumulation of GFP-positive cells in the neuroepithelium and RFP-positive NC cells along the neural plate border, mimicking the TP-0903 treatment (Figure 2.9A and 2.9B; and Movie S10 10). Additionally, we also found RA treatment inhibits expression of EMT regulator *twist1a*, chondrogenic differentiation marker *dlx2a,* and causes an accumulation of pigment cells on the neural tube at 48hpf (Figure 2.9C and 2.9D). These results demonstrate that increased RA is sufficient to impair NC EMT, cell migration and differentiation and indicate the NC phenotypes observed in TP-0903 treated embryos act through increasing RA signaling. Thus, retinoic acid signaling controls multiple events during cranial neural tube development; 1) delamination of NC progenitors within the neural tube, 2) collective cell migration away from the neural tube, and 3) differentiation of ectomesenchymal progenitors. While some of these RA-dependent phenotypes have been observed previously during NC development, these data are the first to show RA directly inhibits EMT *in vivo* and more specifically within the dorsal neural tube during NC development.

Discussion

EMT is essential for embryogenesis, regeneration and cancer metastasis and requires interactions with multiple cell types and ECM components (Lamouille et al., 2014). Modeling the physiological complexity of EMT is not feasible using conventional *in vitro* cell-based systems. Despite this shortcoming, most EMT inhibitors identified to date are discovered through cell-based assays, which likely contributes to the lack of effective EMT inhibitors in the clinic (Davis et al., 2014). In addition, our knowledge of the essential signaling pathways controlling EMT *in vivo* remains incomplete, due to difficulties in labeling and observing EMT in living animals. In this study we take advantage of the unique imaging and *ex utero* development attributes of zebrafish to develop an EMT reporter strain, *Tg(snai1b:GFP),* to directly image EMT in intact animals and identify novel *in vivo* inhibitors of this process. We identified a novel multi-kinase inhibitor, called TP-0903, that functions by activating RA signaling, which in turn blocks EMT and drives differentiation of NC progenitors toward a non-ectomesenchymal fates. Remarkably, despite numerous studies examining RA in NC development, this is the first study showing RA directly controls EMT in NC progenitors during dorsal neural tube development. Finally, as RA is a well-established differentiation agent, these findings also suggest RA antagonizes EMT-dependent pathways controlling de-differentiation and stem-cell-like properties, providing an alternative explanation for the effectiveness of retinoids as adjuvant therapies to eliminate residual tumor cells in cancer patients.

Direct comparative analysis of *Tg(snai1b:GFP)* to previously described NC

reporter strains, such as *sox10*-driven reporters, showed that *Tg(snai1b:GFP)* robustly labels dorsal neural tube progenitors undergoing EMT (Figures 2.1 and 2.3), allowing us to develop assays for identifying inhibitors of EMT using a chemical screening approach. Unexpectedly, we found that inhibition of most canonical kinase and actin-remodeling pathways does not overtly affect EMT and cell migration *in vivo*. We suggest that this is due to multiple compensatory pathways acting together, both within delaminating cells and the microenvironment, to ensure EMT is correctly executed *in vivo*. This contrasts with current 2-D and 3-D *in vitro* assays of EMT that only partially recapitulate the complexities of the microenvironment and thus may be more reliant on single pathways to execute an EMT. Having a complementary *in vivo* model that emulates the full spectrum of EMT is crucial for identifying which *in vitro* EMT inhibitors represent the most effective therapeutics in human disease. In addition, germ-line transgenic lines allow thousands of *Tg(snai1b:GFP)* embryos to be generated by simple genetic crosses for rapid screening purposes. Future studies testing combinations of *in vitro* EMT inhibitors in *Tg(snai1b:GFP)* will likely uncover essential signaling pathways required for EMT *in vivo*, and guide the use of these inhibitors in human disease and cancer.

Early developmental exposure to RA causes overt defects in cranial structures in humans and other vertebrates (Lammer et al., 1985; Lee et al., 1995). Isolated cranial NC cells are highly susceptible to developmental reprograming by RA (Williams et al., 2004) but the consequence of this reprogramming is not known. Interestingly Cyp26c1, an enzyme that oxidizes

and degrades RA to control levels of RA in the neural ectoderm, was established as a novel target of neural plate border specifier genes Pax3 and Zic1 (Plouhinec et al., 2014). Pax3 and Zic1 directly activate a NC gene regulatory network (*snail1*, *snail2*, *foxd3*, and *twist1*) that is sufficient to promote NC specification (Milet et al., 2013; Plouhinec et al., 2014). Together with our data, these findings suggest a model in which Pax3 and Zic1 act to suppress RA signaling at the neural plate border while simultaneously activating *snail1* expression to induce EMT.

Our studies are the first to show RA inhibits cranial NC EMT. There are several ways RA could be blocking EMT. For example RA may activate transcription of epithelial cell-cell adhesion molecules to prevent detachment from the neuroepithelium, as observed in TP-0903 treated embryos (Figure 2.6C). Consistent with this, increased E-cadherin has been observed in NC cells that failed to emigrate from the neural tube in *Cyp26a1/c1*$^{-/-}$ mutant mice (Uehara et al., 2007). Additionally RA treatment increases cytosolic calcium levels (Davis et al., 1991) and cell-cell adhesion of cultured NC cells (Smith-Thomas et al., 1987) and in cancer cell lines RA can activate E-cadherin expression and promote cadherin stabilization at cell membranes (Shah et al., 2002; Woo and Jang, 2012). These observations together with our data suggest excess RA impairs NC EMT and production of migratory NC cells by stimulating Cadherin expression and increasing its localization at cell membranes. The *Tg(snai1b:GFP)* line represents a novel tool to isolate and purify dorsal neural tube progenitors to determine if RA directly regulates Cadherin expression or if

other novel RA-effector pathways inhibit EMT.

Induction of EMT can generate cancer cells with stem-cell-like characteristics and contribute to the formation of a poorly differentiated tumor with increased invasion and metastatic potential. Adjuvant therapies that target dormant/cancer stem cells are already incorporating RA-like molecules into differentiation therapy methods. In the clinic, retinoids are thought to act by promoting an arrest in cell proliferation, inducing differentiation and subsequent cell death (Nasr et al., 2008; Reynolds, 2000). Pharmacological doses of retinoids used in combination with other therapies are being successfully used in the treatment of various types of cancers like neuroblastoma, melanoma, and leukemia (Tang and Gudas, 2011). Our findings provide an alternative viewpoint on retinoid therapy and suggest the positive therapeutic effects of RA observed in the clinic may also be due to its role in blocking EMT. Our data designate RA as an attractive therapeutic approach to both inhibit EMT and promote differentiation of cancer stem cells *in vivo*. However retinoid resistance restricts the clinical benefits of retinoids and continues to be an issue in cancer therapy (Freemantle et al., 2003). The standard use of retinoids involves direct administration into the blood stream, and its effectiveness depends on the cells' ability to transport and/or metabolize exogenous retinoids. The TP-0903 compound identified in this study acts through a novel mechanism that increases intracellular levels of RA by promoting its biosynthesis, which may alleviate retinoid toxicities and resistance. Preclinical studies on established RA-sensitive tumor models will allow assessment of TP-0903 as an alternative adjuvant

therapy to direct RA administration.

Methods

Generating the Tg(snai1b:GFP) construct and transgenic lines

The 3042bp proximal *snai1b* promoter immediately adjacent to the start ATG codon was amplified by standard PCR with primers containing BamH1 (5') and EcoR1 (3') restriction enzyme sites. The amplified fragment was subcloned into a modified *pEGFP-1* plasmid (Clontech) containing I-SceI meganuclease sites flanking the multiple cloning sites and clones verified by restriction digest and sequencing. The *pSnai1b:GFP:Isce1* plasmid was linearized with I-SceI and 10pg of the linearized plasmid was injected into wild-type (AB strain) one-cell zebrafish embryos and monitored for transient GFP expression in the neural tube. GFP-expressing embryos were grown to produce *Tg(snai1b:GFP)* transgenic germ-line founders. The *Tg(snai1b:GFP)*^{zd1100} strain expressed GFP in the same locations as endogenous *snai1b* and used in all subsequent studies (see Figures 2.2 and 2.1).

RNA in situ hybridizations

Embryos were staged by morphological criteria as described (Kimmel et al., 1995). Whole-mount *in situ* hybridizations were carried out as described (Thisse et al., 1993) and antisense probes generated for the following probes: *cyp26a1* (Shelton et al., 2006), *krox20 (Oxtoby and Jowett, 1993)* and *crestin,*

snai1b, dlx2a, and mitfa (Stewart et al., 2006). The *twist1a* cDNA was generated by one-step RT-PCR and cloned into pGEM-T Easy plasmid (Promega) using primers: Forward 5'- GCAATCTGAGCTTTTCCAGAGG- 3', Reverse 5'- ATCCTTATTTTCGCCCTTG- 3'. Antisense *twist1a* probe was generated using T7 polymerase after linearization with Spe1. The *hoxb1a probe* was generated with SP6 polymerase after linearization with EcoRV. Embryos were imaged using a Nikon C-DSD115 microscope with an Olympus DP72 camera. Identical settings were used to obtain *in situ* images within data sets. Brightness and contrast for final images were adjusted identically across data sets using Photoshop CS4.

Time-lapse confocal imaging

Zebrafish embryos were mounted in 35 mm glass bottom microwell dishes with 1% low-melting point agarose in E3 egg water. Mounted embryos were submerged in egg water or egg water containing DMSO, TP-0903, RA and/or DEAB. Time-lapse images were acquired using Olympus Fluoview FV1200 confocal microscope and Olympus FV10-ASW v4.1 software. Olympus UPlanSApo 10X/0.45 objective was used to acquire 10x confocal images every 10-20 minutes and UPlanSApo 60X/1.20W objective was used to acquire 60x confocal images every 35 min.

Chemical compounds and small molecule screening

Tg(snai1b:GFP) zebrafish embryos at ~13 hpf were treated in 12 well plates. One mL of egg water containing pharmacological inhibitors or 0.1-1%

DMSO as control was used to incubate 8-10 embryos/well. Pharmacological inhibitors were removed after 6-12 hrs incubation and embryos screened for EMT and migration defects at 20-24 hpf using fluorescent light on an Olympus SZX16 microscope. TP-0903 (Mollard et al., 2011) was reconstituted to 10 mM in DMSO. All-*trans*-retinoic acid (RA) and Diethylaminobenzaldehyde (DEAB) were purchased from Sigma. Stock solutions of RA (10 mM) and DEAB (10 mM) were prepared in DMSO. All RA treatments were performed in the dark.

Sectioning

Zebrafish embryos were sectioned using a vibratome (Leica VT1200 S) as described (Westerfield, 1993). Embryos were staged and fixed in 4% paraformaldehyde (PFA) at room temperature (RT) for 2 hrs. Fixed embryos were rinsed in 1x phosphate-buffer saline (PBS; pH 7.4) 3x 5 min and then soaked in .3M sucrose dissolved in 1x PBS overnight (O/N) at 4°C. Yolk sacs were removed with forceps before embedding embryos in 17% gelatin dissolved in 10% Hanks Saline at 42°C. Gelatin-tissue blocks were cut into 100um thick sections using vibratome. Sections were then mounted on slides and imaged, or processed and stained as free-floating slices in 12 well plates.

Immunofluorescence

Embryos were fixed in 4% PFA at RT for 2 hrs and sectioned as described above. Slices were rinsed 3x 5 min with .2% Triton-X 100 in PBS and blocked in 1% DMSO, 2mg/ML BSA, .5% Triton-X 100, and 10% normal goat serum in PBS

for 2.5 hrs at RT. Slices were incubated in primary antibody rabbit anti-Cdh1 (1:400, GeneTex), rabbit anti-Pan Cadherin (1:400, Sigma), and mouse anti-GFP (1:400, Clontech Laboratories) O/N at 4°C. Slices were again washed in 1x PBS + .2% Triton-X 100 4x 15 min and blocked for 2.5 hrs at RT. Subsequently slices were incubated in secondary antibody donkey anti-mouse 488 (1:400, Invitrogen), donkey anti-rabbit 568 (1:400, Invitrogen) O/N at 4°C and washed 4x 15 min in PBS + .2% Triton-X 100. Processed sections were mounted on slides and confocal imaging performed using Olympus Fluoview FV1200 confocal microscope with Olympus FV10-ASW v4.1 software.

RNA sequencing

Embryos were treated at 13 hpf (8 somite) with TP-0903 and DMSO for 1-, 4- and 8-hrs at 28°C. 35 embryos were collected for each treatment. Four biological replicates were prepared for each condition. RNA was harvested using Qiagen RNeasy kit (Cat # 74104). Quality of RNA was assessed using Bioanalyzer RNA 6000 Nano Chip and RNA-Seq libraries prepared using illumina TruSeq Stranded Total RNA Sample Prep Kit with Ribo-Zero (Cat # RS-122-2401). Sequencing was performed on Illumina HiSeq 2000 using 50 cycle single-read sequencing v3 kit (Cat # FC-401-3002). Illumina Fatq files were aligned using Novoalign to the zebrafish Zv8 genome. All high-throughput sequencing data sets are currently being submitted to Gene Expression Omnibus (GEO).

References

Amatangelo, M. D., Goodyear, S., Varma, D. and Stearns, M. E. (2012). c-Myc expression and MEK1-induced Erk2 nuclear localization are required for TGF-beta induced epithelial-mesenchymal transition and invasion in prostate cancer. *Carcinogenesis* **33**, 1965-75.

Asiedu, M. K., Beauchamp-Perez, F. D., Ingle, J. N., Behrens, M. D., Radisky, D. C. and Knutson, K. L. (2014). AXL induces epithelial-to-mesenchymal transition and regulates the function of breast cancer stem cells. *Oncogene* **33**, 1316-24.

Banjo, T., Grajcarek, J., Yoshino, D., Osada, H., Miyasaka, K. Y., Kida, Y. S., Ueki, Y., Nagayama, K., Kawakami, K., Matsumoto, T. et al. (2013). Haemodynamically dependent valvulogenesis of zebrafish heart is mediated by flow-dependent expression of miR-21. *Nat Commun* **4**, 1978.

Berndt, J. D., Clay, M. R., Langenberg, T. and Halloran, M. C. (2008). Rho-kinase and myosin II affect dynamic neural crest cell behaviors during epithelial to mesenchymal transition in vivo. *Dev Biol* **324**, 236-44.

Billottet, C., Tuefferd, M., Gentien, D., Rapinat, A., Thiery, J. P., Broet, P. and Jouanneau, J. (2008). Modulation of several waves of gene expression during FGF-1 induced epithelial-mesenchymal transition of carcinoma cells. *J Cell Biochem* **104**, 826-39.

Blackburn, J. S., Liu, S., Wilder, J. L., Dobrinski, K. P., Lobbardi, R., Moore, F. E., Martinez, S. A., Chen, E. Y., Lee, C. and Langenau, D. M. (2014). Clonal evolution enhances leukemia-propagating cell frequency in T cell acute lymphoblastic leukemia through Akt/mTORC1 pathway activation. *Cancer Cell* **25**, 366-78.

Buonato, J. M. and Lazzara, M. J. (2014). ERK1/2 blockade prevents epithelial-mesenchymal transition in lung cancer cells and promotes their sensitivity to EGFR inhibition. *Cancer Res* **74**, 309-19.

Byers, L. A., Diao, L., Wang, J., Saintigny, P., Girard, L., Peyton, M., Shen, L., Fan, Y., Giri, U., Tumula, P. K. et al. (2013). An epithelial-mesenchymal transition gene signature predicts resistance to EGFR and PI3K inhibitors and identifies Axl as a therapeutic target for overcoming EGFR inhibitor resistance. *Clin Cancer Res* **19**, 279-90.

Chou, C. H., Yang, N. K., Liu, T. Y., Tai, S. K., Hsu, D. S., Chen, Y. W., Chen, Y. J., Chang, C. C., Tzeng, C. H. and Yang, M. H. (2013). Chromosome instability modulated by BMI1-AURKA signaling drives progression in head and neck cancer. *Cancer Res* **73**, 953-66.

Cichon, M. A., Szentpetery, Z., Caley, M. P., Papadakis, E. S., Mackenzie, I. C., Brennan, C. H. and O'Toole, E. A. (2014). The receptor tyrosine kinase Axl regulates cell-cell adhesion and stemness in cutaneous squamous cell carcinoma. *Oncogene* **33**, 4185-92.

Clay, M. R. and Halloran, M. C. (2013). Rho activation is apically restricted by Arhgap1 in neural crest cells and drives epithelial-to-mesenchymal transition. *Development* **140**, 3198-209.

Colomiere, M., Ward, A. C., Riley, C., Trenerry, M. K., Cameron-Smith, D., Findlay, J., Ackland, L. and Ahmed, N. (2009). Cross talk of signals between EGFR and IL-6R through JAK2/STAT3 mediate epithelial-mesenchymal transition in ovarian carcinomas. *Br J Cancer* **100**, 134-44.

D'Assoro, A. B., Liu, T., Quatraro, C., Amato, A., Opyrchal, M., Leontovich, A., Ikeda, Y., Ohmine, S., Lingle, W., Suman, V. et al. (2014). The mitotic kinase Aurora--a promotes distant metastases by inducing epithelial-to-mesenchymal transition in ERalpha(+) breast cancer cells. *Oncogene* **33**, 599-610.

Dai, W., Bai, Y., Hebda, L., Zhong, X., Liu, J., Kao, J. and Duan, C. (2014). Calcium deficiency-induced and TRP channel-regulated IGF1R-PI3K-Akt signaling regulates abnormal epithelial cell proliferation. *Cell Death Differ* **21**, 568-81.

Davis, F. M., Stewart, T. A., Thompson, E. W. and Monteith, G. R. (2014). Targeting EMT in cancer: opportunities for pharmacological intervention. *Trends Pharmacol Sci* **35**, 479-88.

Davis, W. L., Jacoby, B. H., Farmer, G. R. and Cooper, O. J. (1991). Changes in cytosolic calcium, bleb formation, and cell death in neural crest cells treated with isotretinoin and 4-oxo-isotretinoin. *J Craniofac Genet Dev Biol* **11**, 105-18.

Delfini, M. C., De La Celle, M., Gros, J., Serralbo, O., Marics, I., Seux, M., Scaal, M. and Marcelle, C. (2009). The timing of emergence of muscle progenitors is controlled by an FGF/ERK/SNAIL1 pathway. *Dev Biol* **333**, 229-37.

Duong, T., Koltowska, K., Pichol-Thievend, C., Le Guen, L., Fontaine, F., Smith, K. A., Truong, V., Skoczylas, R., Stacker, S. A., Achen, M. G. et al. (2014). VEGFD regulates blood vascular development by modulating SOX18 activity. *Blood* **123**, 1102-12.

Ellies, D. L., Langille, R. M., Martin, C. C., Akimenko, M. A. and Ekker, M. (1997). Specific craniofacial cartilage dysmorphogenesis coincides with a loss of dlx gene expression in retinoic acid-treated zebrafish embryos. *Mech Dev* **61**,

23-36.

Erickson, T., French, C. R. and Waskiewicz, A. J. (2010). Meis1 specifies positional information in the retina and tectum to organize the zebrafish visual system. *Neural Dev* **5**, 22.

Ernst, S., Liu, K., Agarwala, S., Moratscheck, N., Avci, M. E., Dalle Nogare, D., Chitnis, A. B., Ronneberger, O. and Lecaudey, V. (2012). Shroom3 is required downstream of FGF signalling to mediate proneuromast assembly in zebrafish. *Development* **139**, 4571-81.

Freemantle, S. J., Spinella, M. J. and Dmitrovsky, E. (2003). Retinoids in cancer therapy and chemoprevention: promise meets resistance. *Oncogene* **22**, 7305-15.

Gjerdrum, C., Tiron, C., Hoiby, T., Stefansson, I., Haugen, H., Sandal, T., Collett, K., Li, S., McCormack, E., Gjertsen, B. T. et al. (2010). Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival. *Proc Natl Acad Sci U S A* **107**, 1124-9.

Green, S. A., Simoes-Costa, M. and Bronner, M. E. (2015). Evolution of vertebrates as viewed from the crest. *Nature* **520**, 474-82.

Grille, S. J., Bellacosa, A., Upson, J., Klein-Szanto, A. J., van Roy, F., Lee-Kwon, W., Donowitz, M., Tsichlis, P. N. and Larue, L. (2003). The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. *Cancer Res* **63**, 2172-8.

Grotegut, S., von Schweinitz, D., Christofori, G. and Lehembre, F. (2006). Hepatocyte growth factor induces cell scattering through MAPK/Egr-1-mediated upregulation of Snail. *EMBO J* **25**, 3534-45.

Gulhati, P., Bowen, K. A., Liu, J., Stevens, P. D., Rychahou, P. G., Chen, M., Lee, E. Y., Weiss, H. L., O'Connor, K. L., Gao, T. et al. (2011). mTORC1 and mTORC2 regulate EMT, motility, and metastasis of colorectal cancer via RhoA and Rac1 signaling pathways. *Cancer Res* **71**, 3246-56.

Gupta, G. P. and Massague, J. (2006). Cancer metastasis: building a framework. *Cell* **127**, 679-95.

Gupta, P. B., Kuperwasser, C., Brunet, J. P., Ramaswamy, S., Kuo, W. L., Gray, J. W., Naber, S. P. and Weinberg, R. A. (2005). The melanocyte differentiation program predisposes to metastasis after neoplastic transformation. *Nat Genet* **37**, 1047-54.

Harding, M. J. and Nechiporuk, A. V. (2012). Fgfr-Ras-MAPK signaling is required for apical constriction via apical positioning of Rho-associated kinase during mechanosensory organ formation. *Development* **139**, 3130-5.

Hardy, K. M., Yatskievych, T. A., Konieczka, J., Bobbs, A. S. and Antin, P. B. (2011). FGF signalling through RAS/MAPK and PI3K pathways regulates cell movement and gene expression in the chicken primitive streak without affecting E-cadherin expression. *BMC Dev Biol* **11**, 20.

Hawkins, T. A., Cavodeassi, F., Erdelyi, F., Szabo, G. and Lele, Z. (2008). The small molecule Mek1/2 inhibitor U0126 disrupts the chordamesoderm to notochord transition in zebrafish. *BMC Dev Biol* **8**, 42.

Hay, E. D. (1995). An overview of epithelio-mesenchymal transformation. *Acta Anat (Basel)* **154**, 8-20.

Holland, S. J., Pan, A., Franci, C., Hu, Y., Chang, B., Li, W., Duan, M., Torneros, A., Yu, J., Heckrodt, T. J. et al. (2010). R428, a selective small molecule inhibitor of Axl kinase, blocks tumor spread and prolongs survival in models of metastatic breast cancer. *Cancer Res* **70**, 1544-54.

Hong, J., Zhou, J., Fu, J., He, T., Qin, J., Wang, L., Liao, L. and Xu, J. (2011). Phosphorylation of serine 68 of Twist1 by MAPKs stabilizes Twist1 protein and promotes breast cancer cell invasiveness. *Cancer Res* **71**, 3980-90.

Irie, H. Y., Pearline, R. V., Grueneberg, D., Hsia, M., Ravichandran, P., Kothari, N., Natesan, S. and Brugge, J. S. (2005). Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. *J Cell Biol* **171**, 1023-34.

Ishizaki, H., Spitzer, M., Wildenhain, J., Anastasaki, C., Zeng, Z., Dolma, S., Shaw, M., Madsen, E., Gitlin, J., Marais, R. et al. (2010). Combined zebrafish-yeast chemical-genetic screens reveal gene-copper-nutrition interactions that modulate melanocyte pigmentation. *Dis Model Mech* **3**, 639-51.

Kang, Y. and Massague, J. (2004). Epithelial-mesenchymal transitions: twist in development and metastasis. *Cell* **118**, 277-9.

Kim, E., Youn, H., Kwon, T., Son, B., Kang, J., Yang, H. J., Seong, K. M., Kim, W. and Youn, B. (2014). PAK1 tyrosine phosphorylation is required to induce epithelial-mesenchymal transition and radioresistance in lung cancer cells. *Cancer Res* **74**, 5520-31.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev Dyn* **203**, 253-310.

Knecht, A. K. and Bronner-Fraser, M. (2002). Induction of the neural crest: a multigene process. *Nat Rev Genet* **3**, 453-61.

Kong, F. F., Zhu, Y. L., Yuan, H. H., Wang, J. Y., Zhao, M., Gong, X. D., Liu, F., Zhang, W. Y., Wang, C. R. and Jiang, B. (2014). FOXM1 regulated by ERK pathway mediates TGF-beta1-induced EMT in NSCLC. *Oncol Res* **22**, 29-37.

Koorstra, J. B., Karikari, C. A., Feldmann, G., Bisht, S., Rojas, P. L., Offerhaus, G. J., Alvarez, H. and Maitra, A. (2009). The Axl receptor tyrosine kinase confers an adverse prognostic influence in pancreatic cancer and represents a new therapeutic target. *Cancer Biol Ther* **8**, 618-26.

Kurrey, N. K., Jalgaonkar, S. P., Joglekar, A. V., Ghanate, A. D., Chaskar, P. D., Doiphode, R. Y. and Bapat, S. A. (2009). Snail and slug mediate radioresistance and chemoresistance by antagonizing p53-mediated apoptosis and acquiring a stem-like phenotype in ovarian cancer cells. *Stem Cells* **27**, 2059-68.

Lammer, E. J., Chen, D. T., Hoar, R. M., Agnish, N. D., Benke, P. J., Braun, J. T., Curry, C. J., Fernhoff, P. M., Grix, A. W., Jr., Lott, I. T. et al. (1985). Retinoic acid embryopathy. *N Engl J Med* **313**, 837-41.

Lamouille, S., Connolly, E., Smyth, J. W., Akhurst, R. J. and Derynck, R. (2012). TGF-beta-induced activation of mTOR complex 2 drives epithelial-mesenchymal transition and cell invasion. *J Cell Sci* **125**, 1259-73.

Lamouille, S. and Derynck, R. (2007). Cell size and invasion in TGF-beta-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. *J Cell Biol* **178**, 437-51.

Lamouille, S., Xu, J. and Derynck, R. (2014). Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* **15**, 178-96.

Latifi, A., Abubaker, K., Castrechini, N., Ward, A. C., Liongue, C., Dobill, F., Kumar, J., Thompson, E. W., Quinn, M. A., Findlay, J. K. et al. (2011). Cisplatin treatment of primary and metastatic epithelial ovarian carcinomas generates residual cells with mesenchymal stem cell-like profile. *J Cell Biochem* **112**, 2850-64.

Lee, Y. M., Osumi-Yamashita, N., Ninomiya, Y., Moon, C. K., Eriksson, U. and Eto, K. (1995). Retinoic acid stage-dependently alters the migration pattern and identity of hindbrain neural crest cells. *Development* **121**, 825-37.

Letamendia, A., Quevedo, C., Ibarbia, I., Virto, J. M., Holgado, O., Diez, M., Izpisua Belmonte, J. C. and Callol-Massot, C. (2012). Development

and validation of an automated high-throughput system for zebrafish in vivo screenings. *PLoS One* **7**, e36690.

Li, Q. Q., Xu, J. D., Wang, W. J., Cao, X. X., Chen, Q., Tang, F., Chen, Z. Q., Liu, X. P. and Xu, Z. D. (2009). Twist1-mediated adriamycin-induced epithelial-mesenchymal transition relates to multidrug resistance and invasive potential in breast cancer cells. *Clin Cancer Res* **15**, 2657-65.

Lin, J., Wang, X. and Dorsky, R. I. (2011). Progenitor expansion in apc mutants is mediated by Jak/Stat signaling. *BMC Dev Biol* **11**, 73.

Liu, L., Zhu, S., Gong, Z. and Low, B. C. (2008). K-ras/PI3K-Akt signaling is essential for zebrafish hematopoiesis and angiogenesis. *PLoS One* **3**, e2850.

Liu, R. Y., Zeng, Y., Lei, Z., Wang, L., Yang, H., Liu, Z., Zhao, J. and Zhang, H. T. (2014). JAK/STAT3 signaling is required for TGF-beta-induced epithelial-mesenchymal transition in lung cancer cells. *Int J Oncol* **44**, 1643-51.

Lo, H. W., Hsu, S. C., Xia, W., Cao, X., Shih, J. Y., Wei, Y., Abbruzzese, J. L., Hortobagyi, G. N. and Hung, M. C. (2007). Epidermal growth factor receptor cooperates with signal transducer and activator of transcription 3 to induce epithelial-mesenchymal transition in cancer cells via up-regulation of TWIST gene expression. *Cancer Res* **67**, 9066-76.

Lu, K. V., Chang, J. P., Parachoniak, C. A., Pandika, M. M., Aghi, M. K., Meyronet, D., Isachenko, N., Fouse, S. D., Phillips, J. J., Cheresh, D. A. et al. (2012). VEGF inhibits tumor cell invasion and mesenchymal transition through a MET/VEGFR2 complex. *Cancer Cell* **22**, 21-35.

Lu, Z., Ghosh, S., Wang, Z. and Hunter, T. (2003). Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion. *Cancer Cell* **4**, 499-515.

Luesch, H., Chanda, S. K., Raya, R. M., DeJesus, P. D., Orth, A. P., Walker, J. R., Izpisua Belmonte, J. C. and Schultz, P. G. (2006). A functional genomics approach to the mode of action of apratoxin A. *Nat Chem Biol* **2**, 158-67.

Ma, A. C., Fan, A., Ward, A. C., Liongue, C., Lewis, R. S., Cheng, S. H., Chan, P. K., Yip, S. F., Liang, R. and Leung, A. Y. (2009). A novel zebrafish jak2a(V581F) model shared features of human JAK2(V617F) polycythemia vera. *Exp Hematol* **37**, 1379-1386 e4.

Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A.

Y., Brooks, M., Reinhard, F., Zhang, C. C., Shipitsin, M. et al. (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133**, 704-15.

Marchetti, A., Colletti, M., Cozzolino, A. M., Steindler, C., Lunadei, M., Mancone, C. and Tripodi, M. (2008). ERK5/MAPK is activated by TGFbeta in hepatocytes and required for the GSK-3beta-mediated Snail protein stabilization. *Cell Signal* **20**, 2113-8.

Martinez-Morales, P. L., Diez del Corral, R., Olivera-Martinez, I., Quiroga, A. C., Das, R. M., Barbas, J. A., Storey, K. G. and Morales, A. V. (2011). FGF and retinoic acid activity gradients control the timing of neural crest cell emigration in the trunk. *J Cell Biol* **194**, 489-503.

Matt, N., Ghyselinck, N. B., Wendling, O., Chambon, P. and Mark, M. (2003). Retinoic acid-induced developmental defects are mediated by RARbeta/RXR heterodimers in the pharyngeal endoderm. *Development* **130**, 2083-93.

Milet, C., Maczkowiak, F., Roche, D. D. and Monsoro-Burq, A. H. (2013). Pax3 and Zic1 drive induction and differentiation of multipotent, migratory, and functional neural crest in Xenopus embryos. *Proc Natl Acad Sci U S A* **110**, 5528-33.

Molina, G. A., Watkins, S. C. and Tsang, M. (2007). Generation of FGF reporter transgenic zebrafish and their utility in chemical screens. *BMC Dev Biol* **7**, 62.

Mollard, A., Warner, S. L., Call, L. T., Wade, M. L., Bearss, J. J., Verma, A., Sharma, S., Vankayalapati, H. and Bearss, D. J. (2011). Design, synthesis and biological evaluation of a series of novel Axl kinase inhibitors. *ACS Med Chem Lett* **2**, 907-912.

Nasr, R., Guillemin, M. C., Ferhi, O., Soilihi, H., Peres, L., Berthier, C., Rousselot, P., Robledo-Sarmiento, M., Lallemand-Breitenbach, V., Gourmel, B. et al. (2008). Eradication of acute promyelocytic leukemia-initiating cells through PML-RARA degradation. *Nat Med* **14**, 1333-42.

Nguyen, P. T., Tsunematsu, T., Yanagisawa, S., Kudo, Y., Miyauchi, M., Kamata, N. and Takata, T. (2013). The FGFR1 inhibitor PD173074 induces mesenchymal-epithelial transition through the transcription factor AP-1. *Br J Cancer* **109**, 2248-58.

Nieto, M. A. (2002). The snail superfamily of zinc-finger transcription factors. *Nat Rev Mol Cell Biol* **3**, 155-66.

Niu, N. K., Wang, Z. L., Pan, S. T., Ding, H. Q., Au, G. H., He, Z. X., Zhou, Z. W., Xiao, G., Yang, Y. X., Zhang, X. et al. (2015). Pro-apoptotic and pro-autophagic effects of the Aurora kinase A inhibitor alisertib (MLN8237) on human osteosarcoma U-2 OS and MG-63 cells through the activation of mitochondria-mediated pathway and inhibition of p38 MAPK/PI3K/Akt/mTOR signaling pathway. *Drug Des Devel Ther* **9**, 1555-84.

Oxtoby, E. and Jowett, T. (1993). Cloning of the zebrafish krox-20 gene (krx-20) and its expression during hindbrain development. *Nucleic Acids Res* **21**, 1087-95.

Ozdamar, B., Bose, R., Barrios-Rodiles, M., Wang, H. R., Zhang, Y. and Wrana, J. L. (2005). Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. *Science* **307**, 1603-9.

Pennisi, D. J. and Mikawa, T. (2009). FGFR-1 is required by epicardium-derived cells for myocardial invasion and correct coronary vascular lineage differentiation. *Dev Biol* **328**, 148-59.

Plouhinec, J. L., Roche, D. D., Pegoraro, C., Figueiredo, A. L., Maczkowiak, F., Brunet, L. J., Milet, C., Vert, J. P., Pollet, N., Harland, R. M. et al. (2014). Pax3 and Zic1 trigger the early neural crest gene regulatory network by the direct activation of multiple key neural crest specifiers. *Dev Biol* **386**, 461-72.

Polyak, K. and Weinberg, R. A. (2009). Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer* **9**, 265-73.

Pruvot, B., Cure, Y., Djiotsa, J., Voncken, A. and Muller, M. (2014). Developmental defects in zebrafish for classification of EGF pathway inhibitors. *Toxicol Appl Pharmacol* **274**, 339-49.

Qian, X., Anzovino, A., Kim, S., Suyama, K., Yao, J., Hulit, J., Agiostratidou, G., Chandiramani, N., McDaid, H. M., Nagi, C. et al. (2014). N-cadherin/FGFR promotes metastasis through epithelial-to-mesenchymal transition and stem/progenitor cell-like properties. *Oncogene* **33**, 3411-21.

Reichl, P., Dengler, M., van Zijl, F., Huber, H., Fuhrlinger, G., Reichel, C., Sieghart, W., Peck-Radosavljevic, M., Grubinger, M. and Mikulits, W. (2015). Axl activates autocrine transforming growth factor-beta signaling in hepatocellular carcinoma. *Hepatology* **61**, 930-41.

Reynolds, C. P. (2000). Differentiating agents in pediatric malignancies: retinoids in neuroblastoma. *Curr Oncol Rep* **2**, 511-8.

Ronca, R., Di Salle, E., Giacomini, A., Leali, D., Alessi, P., Coltrini, D., Ravelli, C., Matarazzo, S., Ribatti, D., Vermi, W. et al. (2013). Long pentraxin-3 inhibits epithelial-mesenchymal transition in melanoma cells. *Mol Cancer Ther* **12**, 2760-71.

Samame Perez-Vargas, J. C., Biondani, P., Maggi, C., Gariboldi, M., Gloghini, A., Inno, A., Volpi, C. C., Gualeni, A. V., di Bartolomeo, M., de Braud, F. et al. (2013). Role of cMET in the development and progression of colorectal cancer. *Int J Mol Sci* **14**, 18056-77.

Sauka-Spengler, T. and Bronner-Fraser, M. (2008). A gene regulatory network orchestrates neural crest formation. *Nat Rev Mol Cell Biol* **9**, 557-68.

Savagner, P., Yamada, K. M. and Thiery, J. P. (1997). The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition. *J Cell Biol* **137**, 1403-19.

Shah, S., Pishvaian, M. J., Easwaran, V., Brown, P. H. and Byers, S. W. (2002). The role of cadherin, beta-catenin, and AP-1 in retinoid-regulated carcinoma cell differentiation and proliferation. *J Biol Chem* **277**, 25313-22.

Shelton, D. N., Sandoval, I. T., Eisinger, A., Chidester, S., Ratnayake, A., Ireland, C. M. and Jones, D. A. (2006). Up-regulation of CYP26A1 in adenomatous polyposis coli-deficient vertebrates via a WNT-dependent mechanism: implications for intestinal cell differentiation and colon tumor development. *Cancer Res* **66**, 7571-7.

Shen, X., Li, J., Hu, P. P., Waddell, D., Zhang, J. and Wang, X. F. (2001). The activity of guanine exchange factor NET1 is essential for transforming growth factor-beta-mediated stress fiber formation. *J Biol Chem* **276**, 15362-8.

Silva, S. R., Bowen, K. A., Rychahou, P. G., Jackson, L. N., Weiss, H. L., Lee, E. Y., Townsend, C. M., Jr. and Evers, B. M. (2011). VEGFR-2 expression in carcinoid cancer cells and its role in tumor growth and metastasis. *Int J Cancer* **128**, 1045-56.

Smith-Thomas, L., Lott, I. and Bronner-Fraser, M. (1987). Effects of isotretinoin on the behavior of neural crest cells in vitro. *Dev Biol* **123**, 276-81.

Stewart, R. A., Arduini, B. L., Berghmans, S., George, R. E., Kanki, J. P., Henion, P. D. and Look, A. T. (2006). Zebrafish foxd3 is selectively required for neural crest specification, migration and survival. *Dev Biol* **292**, 174-88.

Sun, X., Meyers, E. N., Lewandoski, M. and Martin, G. R. (1999).

Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. *Genes Dev* **13**, 1834-46.

Tang, X. H. and Gudas, L. J. (2011). Retinoids, retinoic acid receptors, and cancer. *Annu Rev Pathol* **6**, 345-64.

Tavares, A. L., Mercado-Pimentel, M. E., Runyan, R. B. and Kitten, G. T. (2006). TGF beta-mediated RhoA expression is necessary for epithelial-mesenchymal transition in the embryonic chick heart. *Dev Dyn* **235**, 1589-98.

Thiery, J. P., Acloque, H., Huang, R. Y. and Nieto, M. A. (2009). Epithelial-mesenchymal transitions in development and disease. *Cell* **139**, 871-90.

Thisse, C., Thisse, B., Schilling, T. F. and Postlethwait, J. H. (1993). Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. *Development* **119**, 1203-15.

Tolero Pharmaceuticals. (2015). TP-0903, vol. 2015: [Internet] http://www.toleropharmaceuticals.com/TP-0903.html.

Uehara, M., Yashiro, K., Mamiya, S., Nishino, J., Chambon, P., Dolle, P. and Sakai, Y. (2007). CYP26A1 and CYP26C1 cooperatively regulate anterior-posterior patterning of the developing brain and the production of migratory cranial neural crest cells in the mouse. *Dev Biol* **302**, 399-411.

Valles, A. M., Boyer, B., Badet, J., Tucker, G. C., Barritault, D. and Thiery, J. P. (1990). Acidic fibroblast growth factor is a modulator of epithelial plasticity in a rat bladder carcinoma cell line. *Proc Natl Acad Sci U S A* **87**, 1124-8.

Valles, A. M., Boyer, B., Tarone, G. and Thiery, J. P. (1996). Alpha 2 beta 1 integrin is required for the collagen and FGF-1 induced cell dispersion in a rat bladder carcinoma cell line. *Cell Adhes Commun* **4**, 187-99.

Vardouli, L., Moustakas, A. and Stournaras, C. (2005). LIM-kinase 2 and cofilin phosphorylation mediate actin cytoskeleton reorganization induced by transforming growth factor-beta. *J Biol Chem* **280**, 11448-57.

Verma, N., Keinan, O., Selitrennik, M., Karn, T., Filipits, M. and Lev, S. (2015). PYK2 sustains endosomal-derived receptor signalling and enhances epithelial-to-mesenchymal transition. *Nat Commun* **6**, 6064.

Vuoriluoto, K., Haugen, H., Kiviluoto, S., Mpindi, J. P., Nevo, J., Gjerdrum, C., Tiron, C., Lorens, J. B. and Ivaska, J. (2011). Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by

governing Axl expression in breast cancer. *Oncogene* **30**, 1436-48.

Wan, X. B., Long, Z. J., Yan, M., Xu, J., Xia, L. P., Liu, L., Zhao, Y., Huang, X. F., Wang, X. R., Zhu, X. F. et al. (2008). Inhibition of Aurora-A suppresses epithelial-mesenchymal transition and invasion by downregulating MAPK in nasopharyngeal carcinoma cells. *Carcinogenesis* **29**, 1930-7.

Weiser, D. C., Pyati, U. J. and Kimelman, D. (2007). Gravin regulates mesodermal cell behavior changes required for axis elongation during zebrafish gastrulation. *Genes Dev* **21**, 1559-71.

Westerfield, M. (1993). The zebrafish book : a guide for the laboratory use of zebrafish (Brachydanio rerio). Eugene, OR: M. Westerfield.

Williams, S. S., Mear, J. P., Liang, H. C., Potter, S. S., Aronow, B. J. and Colbert, M. C. (2004). Large-scale reprogramming of cranial neural crest gene expression by retinoic acid exposure. *Physiol Genomics* **19**, 184-97.

Woo, Y. J. and Jang, K. L. (2012). All-trans retinoic acid activates E-cadherin expression via promoter hypomethylation in the human colon carcinoma HCT116 cells. *Biochem Biophys Res Commun* **425**, 944-9.

Xie, L., Law, B. K., Chytil, A. M., Brown, K. A., Aakre, M. E. and Moses, H. L. (2004). Activation of the Erk pathway is required for TGF-beta1-induced EMT in vitro. *Neoplasia* **6**, 603-10.

Yadav, A., Kumar, B., Datta, J., Teknos, T. N. and Kumar, P. (2011). IL-6 promotes head and neck tumor metastasis by inducing epithelial-mesenchymal transition via the JAK-STAT3-SNAIL signaling pathway. *Mol Cancer Res* **9**, 1658-67.

Yang, J., Mani, S. A., Donaher, J. L., Ramaswamy, S., Itzykson, R. A., Come, C., Savagner, P., Gitelman, I., Richardson, A. and Weinberg, R. A. (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* **117**, 927-39.

Yu, L., Hebert, M. C. and Zhang, Y. E. (2002). TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. *EMBO J* **21**, 3749-59.

Zhang, Z., Lee, J. C., Lin, L., Olivas, V., Au, V., LaFramboise, T., Abdel-Rahman, M., Wang, X., Levine, A. D., Rho, J. K. et al. (2012). Activation of the AXL kinase causes resistance to EGFR-targeted therapy in lung cancer. *Nat Genet* **44**, 852-60.

Zheng, H. X., Cai, Y. D., Wang, Y. D., Cui, X. B., Xie, T. T., Li, W. J.,

Peng, L., Zhang, Y., Wang, Z. Q., Wang, J. et al. (2013). Fas signaling promotes motility and metastasis through epithelial-mesenchymal transition in gastrointestinal cancer. *Oncogene* **32**, 1183-92.

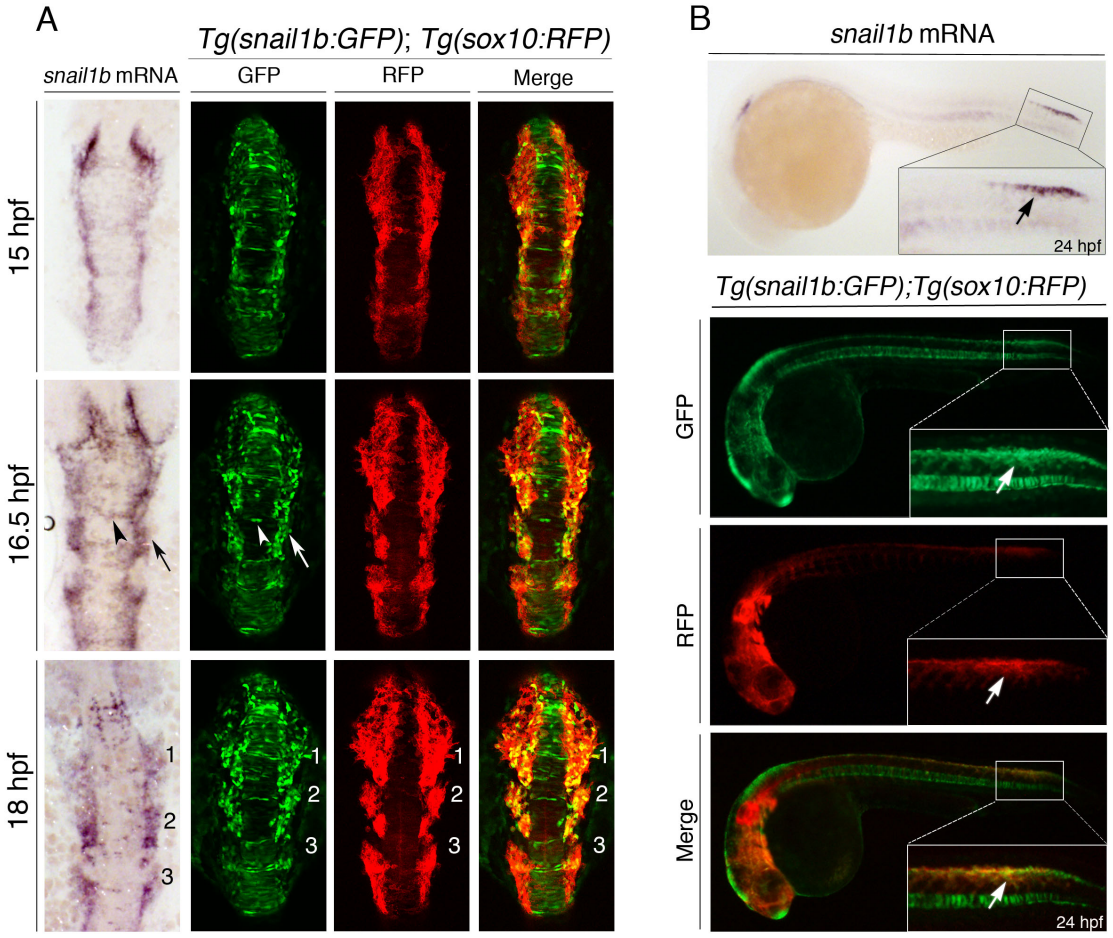


Figure 2.1. Generating an NC EMT reporter during development. (A) Dorsal views of whole mount *in situ* hybridization of *snail1b mRNA* expression (left) compared to maximal z-projections of double transgenic *Tg(snai1b:GFP); Tg(sox10:RFP)* zebrafish embryos (right) at indicated developmental time points. *Tg(snai1b:GFP)* expresses GFP in the neuroepithelium (white arrow head) and neural plate border cells (white arrow), similar to endogenous *snail1b* mRNA expression (black arrow and arrowhead). Comparison of *Tg(snai1b:GFP)* with *Tg(sox10:RFP)* shows GFP is expressed in both the neuroepithelium and lateral plate border, whereas RFP labels delaminating NC cells at the neural plate border, and partially overlaps with GFP (yellow cells). In migrating NC cells, GFP and RFP are co-expressed in the first two cranial streams (numbered). (B) Lateral views of brightfield (top) or epifluorescent images (bottom) of 24 hpf embryos comparing *snail1b* mRNA *in situ* hybridization expression in the trunk NC and *Tg(snai1b:GFP);Tg(sox10:RFP)* embryos showing *Tg(snai1b:GFP)* labels the same region as endogenous *snail1b* mRNA and co-labels with *sox10:RFP* in trunk NC cells (white arrows).

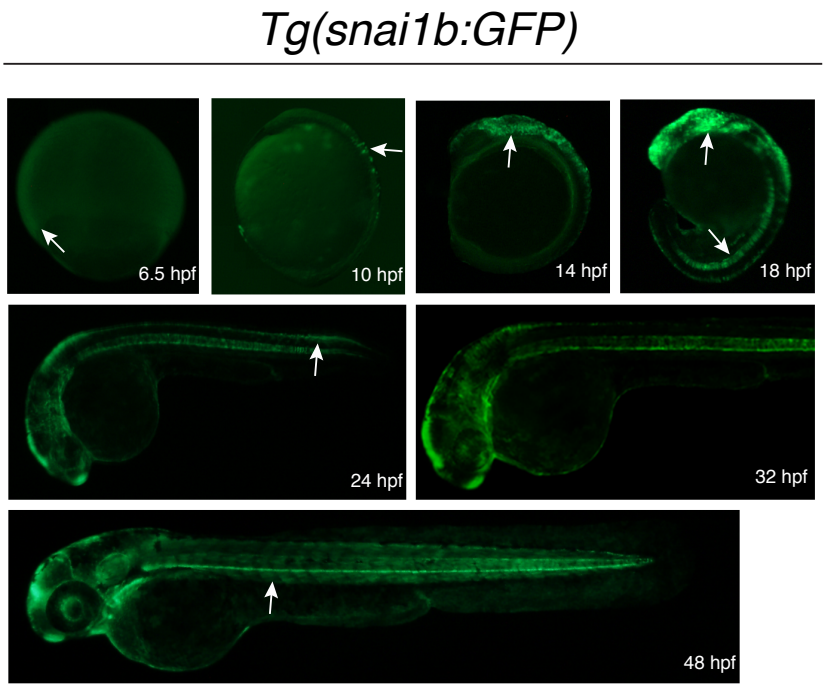


Figure 2.2. Developmental expression of *Tg(snai1b:GFP).* (A) *Tg(snai1b:GFP)* labels cells during gastrulation at 6.5 hpf in involuting mesendoderm cells (arrow), (B) and within the anterior portion of the neuroepithelium (arrow) at 10 hpf. (C) When cranial NC EMT is beginning to initiate at 14 hpf GFP positive cells are found in the neuroepithelium and along the neural plate border (arrow). (D) By 18 hpf GFP expression can be seen in the notochord and within cranial neural crest streams (arrows). (E) At 24 hpf *Tg(snai1b:GFP)* labels trunk NC cells undergoing EMT and (G) somites undergoing EMT are also labeled at 48 hpf.

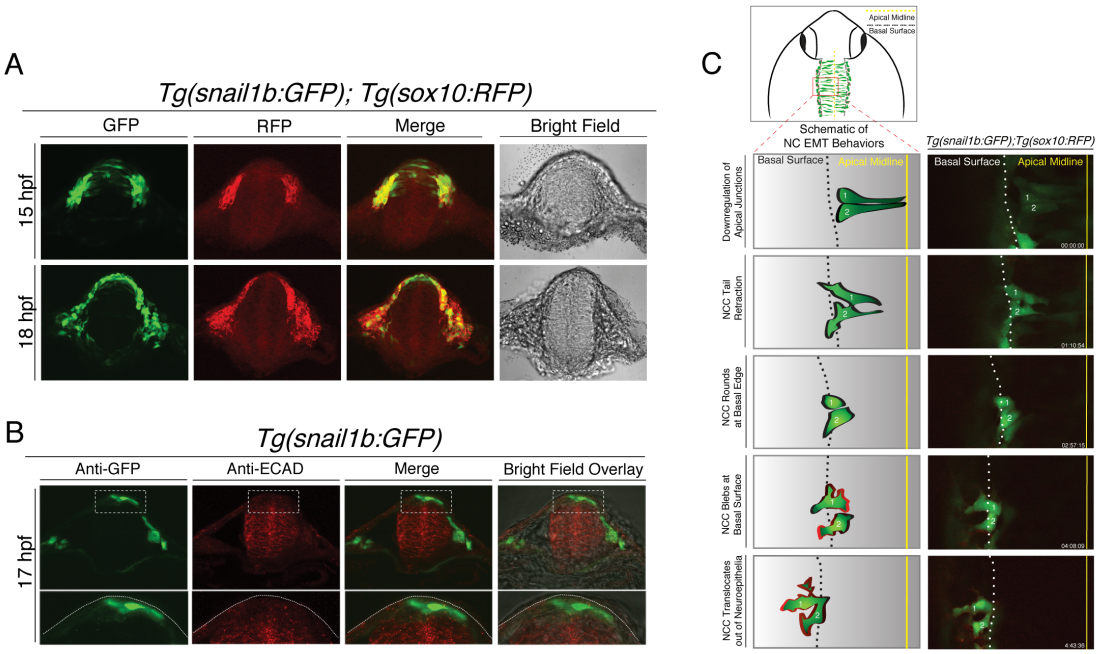


Figure 2.3. *Tg(snai1b:GFP)* labels dorsal neural tube progenitors that display morphological cell behaviors of EMT. (A) Transverse hindbrain sections in double transgenic *Tg(snai1b:GFP); Tg(sox10:RFP)* embryos at 15 and 18 hpf (single confocal z plane, 40x) showing *snai1b*-driven GFP is expressed in dorsal neural tube progenitor cells at 15 hpf, while both GFP and *sox10*-driven RFP are expressed in cells adjacent to the neural tube and migration NC cells. (B) Transverse hindbrain sections of *Tg(snai1b:GFP)* embryos at 17 hpf processed for immunofluorescence analysis of a-Cdh1 (ECAD) and GFP. Bottom row displays higher magnification views of boxed regions, and shows decreased ECAD levels in GFP-positive cells compared to ventral neural tube. (C) Schematic illustrating imaging area (top) and cell delamination behavior (bottom left) derived from confocal time-lapse images of *Tg(snai1b:GFP); Tg(sox10:RFP)* embryos (bottom right), which captured 2 cells delaminating out of the neuroepithelium to produce *sox10*-positive NC cells.

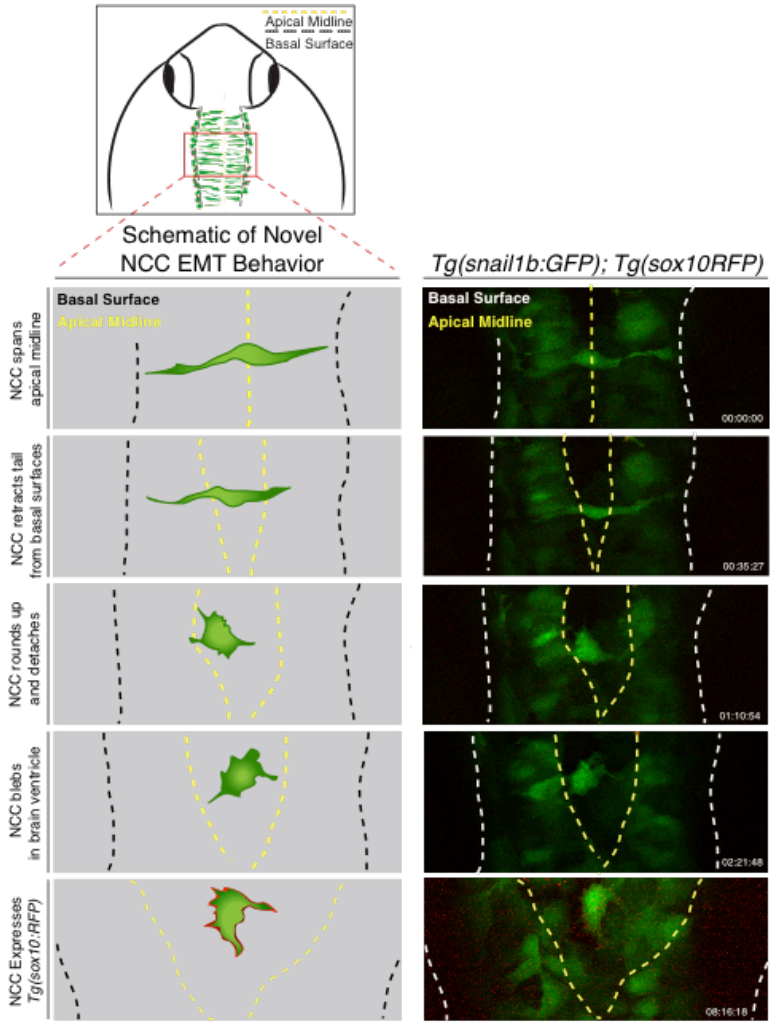


Figure 2.4. Dorsal midline neural epithelial progenitor cells display novel delamination behaviors of EMT. Schematic illustrating imaging area (top) and novel delamination behavior of a dorsal midline neuroepithelial progenitor (left), which is derived from confocal time-lapse images of *Tg(snai1b:GFP); Tg(sox10:RFP)* embryos (right). Images show a GFP-positive cell initially spanning the dorsal midline before contracting apical attachments from each side of neuroepithelium. Subsequently, GFP-positive cells detach and become rounded with filopodia and blebbing protrusions, and ultimately express *sox10-mRFP* at cell membranes. See also Movie S3.

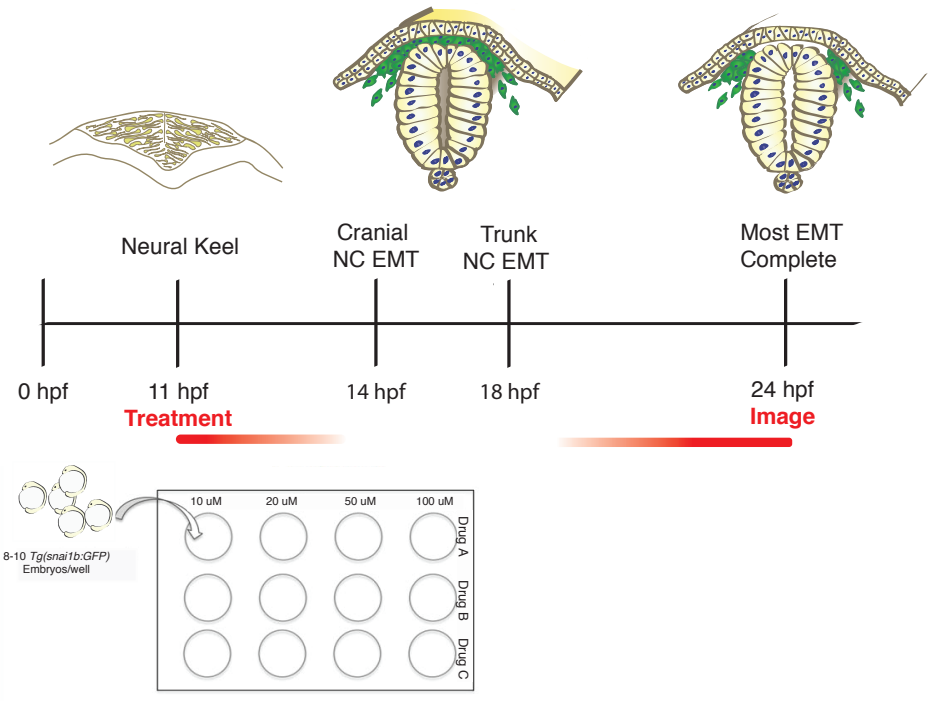


Figure 2.5. Small molecule screening assay to identify inhibitors of EMT and cell migration using *Tg(snai1b:GFP)* zebrafish embryos. *Tg(snai1b:GFP)* embryos were treated after NC induction at ~11 hpf in 12 well plates (see Methods). Embryos were incubated at 28°C with pharmacological inhibitors or DMSO for 12-13 hrs and visualized at 24 hpf after majority of cranial NC EMT has been completed and migration initiated. Pharmacological inhibitors that affect EMT and cell migration display an accumulation of GFP-positive cells within the neural tube and adjacent to the neural plate border.

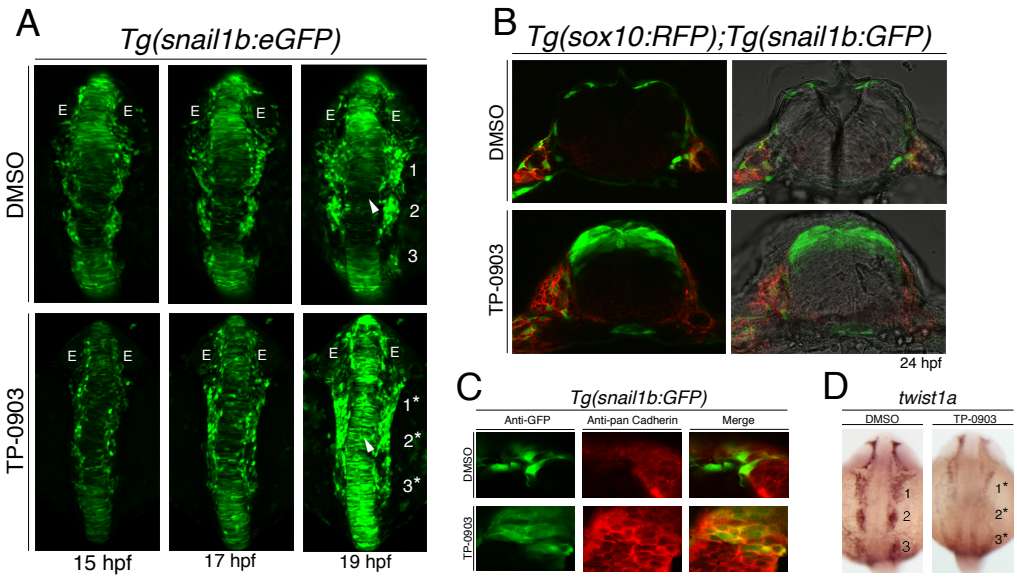


Figure 2.6. Chemical screening with *Tg(snai1b:GFP)* identifies TP-0903 as an inhibitor of NC EMT. (A) Dorsal views of *Tg(snai1b:GFP)* embryos treated with DMSO (top) or TP-0903 (bottom) at 11.5 hpf and imaged at indicated time points by 10x confocal time-lapse microscopy. TP-0903 treated embryos accumulate GFP-positive cells in the dorsal neural tube (arrowhead) and lateral cells remain associated with the neural plate border and are fused (compare numbered streams in DMSO control to numbered asterisks in TP-0903). (B) Transverse sections of DMSO (top) and TP-0903 treated (bottom) *Tg(snai1b:GFP); Tg(sox10:RFP)* embryos at 24 hpf, showing TP-0903 causes accumulation of GFP-positive cells in the dorsal neural tube. (C) Transverse sections through hindbrain of *Tg(snai1b:GFP)* embryos at 24 hpf treated with DMSO (top) and TP-0903 (bottom) and analyzed by immunofluorescence for a-pan-Cadherin and GFP, showing GFP-positive cells in TP-0903 treated embryos maintain Cadherin levels and cell-cell adhesion within the neural tube. (D) Dorsal views of DMSO (left) and TP-0903 treated (right) embryos at 18 hpf processed for *twist1a* mRNA *in situ* hybridization showing TP-0903 significantly reduces *twist1a* expression levels (compare numbered streams in DMSO to numbered asterisks in TP-0903).

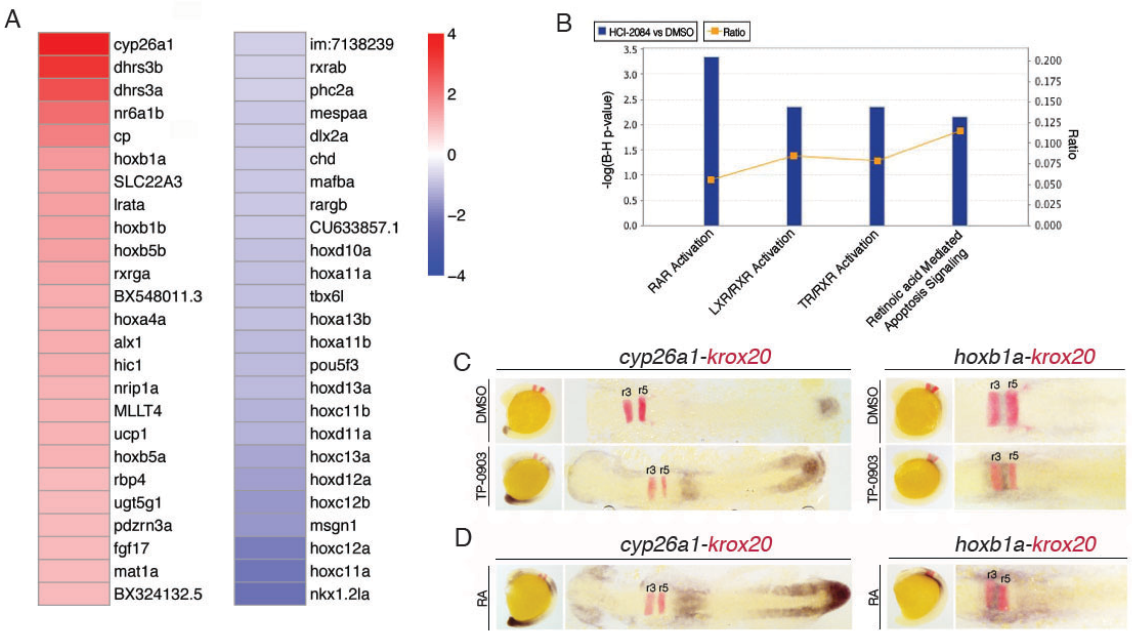


Figure 2.7. TP-0903 acts by inducing retinoic acid signaling in zebrafish embryos. (A) Heat map schematic showing the 50 most differentially expressed transcripts from RNA-Seq as measured by log2 ratio from 4 hr treated DMSO and TP-0903 embryos. (B) Ingenuity pathway analysis of differentially expressed genes in TP-0903 treated embryos reveals RA signaling pathways are exclusively activated. Bars represent the p-value for each RA pathway (expressed as -1 X log of the p-value). The yellow line represents the ratio of the number of genes from our data set represented within each pathway to the total number of genes in each pathway. (C) Lateral (left panels) and dorsal flat-mounted views (right panels) of *in situ* hybridization at 16 hpf for two genes, *cyp26a1* and *hoxb1a,* identified as elevated by RNA-Seq after 4 hr TP-0903 treatment. In all panels *krox20* (red) is used as a marker of rhombomeres r3 and r5 (red stain). (D) Lateral (left panels) and dorsal flat-mounted views (right panels) of embryos treated at 13 hpf with RA mimic TP-0903 expression effects on *cyp26a1* and *hoxb1a*.

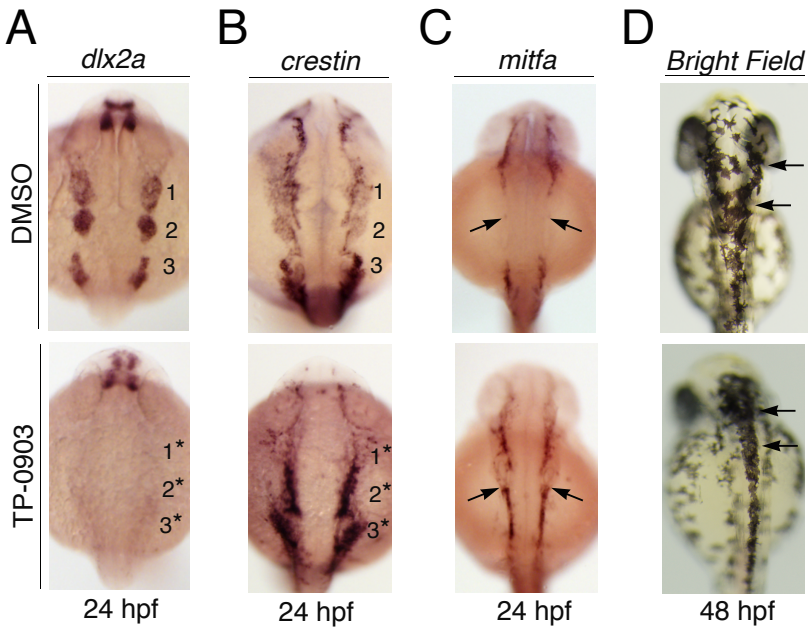


Figure 2.8. TP-0903 blocks ectomesenchyme differentiation and promotes melanophore differentiation. Dorsal views of embryos treated with DMSO or TP-0903 at 3-somite stage and fixed at the indicated time points for processing by whole mount *in situ* hybridization and bright field microscopy with indicated probes or direct visualization of pigment cells. (A) Compared to controls (top panel), TP-0903 completely inhibits *dlx2a* expression in cranial NC streams (n= 24). (B) Analysis of TP-0903 treated embryos with the pan-NC marker *crestin* shows NC cells are induced but fail to migrate away, and the first two cranial NC streams are fused together, as indicated by numbered asterisks. (C) Stalled NC cells in TP-0903 treated embryos express the melanophore precursor marker *mitfa* (black arrows). (D) Conversion of ectomesenchymal cells to non-ectomesenchymal cells causes the accumulation of melanophores on the dorsal neural tube at 48 hpf.

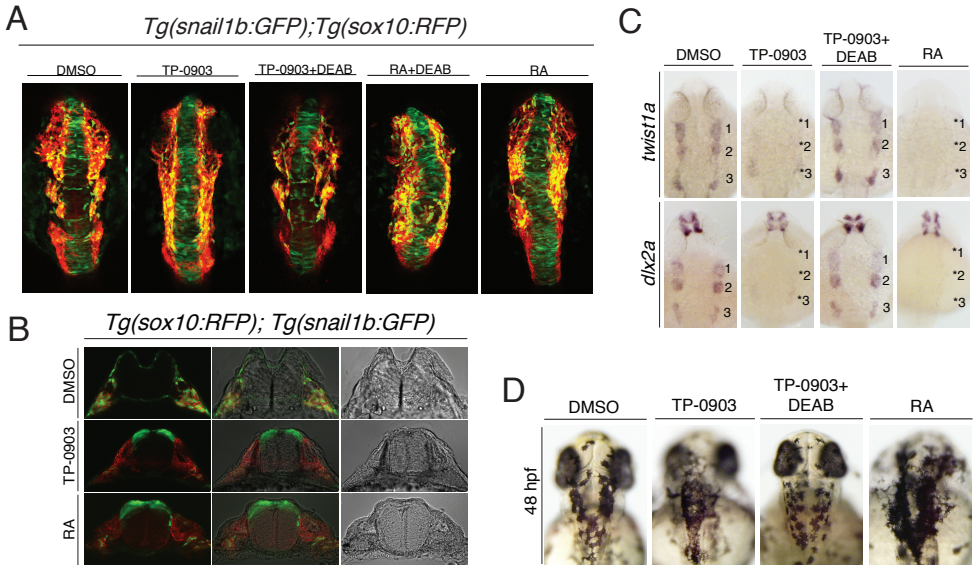


Figure 2.9. Retinoic acid controls cranial NC EMT, cell migration, and ectomesenchyme differentiation. (A) Dorsal views of maximal z-projection confocal images of *Tg(snai1b:GFP); Tg(sox10:RFP)* embryos treated with indicated compounds at 11.5 hpf. TP-0903 inhibits NC EMT and cell migration, which is rescued by co-treatment with DEAB, an inhibitor of retinaldehyde dehydrogenase required for retinoic acid biosynthesis. In contrast, direct treatment with RA bypasses the requirement for retinaldehyde dehydrogenase and is not rescued by DEAB. (B) Transverse sections through the hindbrain of *Tg(snail1b); Tg(sox10:RFP)* embryos at 24 hpf treated with DMSO, TP-0903 and RA confirms RA treatment causes accumulation of GFP-positive dorsal neural tube cells similar to TP-0903 treatment. (C) Dorsal views of 24 hpf embryos processed by whole mount *in situ* hybridization for *twist1a* and *dlx2a* mRNA showing inhibition of RA synthesis in TP-0903 treated embryos rescues expression of these genes in NC streams (numbered), while RA treatment itself mimics TP-0903 and blocks expression of *twist1a* and *dlx2a.* (D) Dorsal views of 48 hpf embryos showing melanophores accumulate on the head of TP-0903 treated embryos, which is rescued by DEAB co-treatment and mimicked by direct RA treatment.

Table 2.1. Small molecules screened in *Tg(snai1b:GFP)* embryos to identify *in vivo* regulators of EMT and cell migration. Initial concentrations of 10 uM, 20 uM, 50 uM, and 100 uM where tested for all compounds.

Pharmacological Inhibitors	Target	Inhibits EMT *In Vitro*	Inhibits EMT *In Vivo*	Target Linked to EMT	Tested in Zebrafish
TG101209	JAK2	(Kim et al., 2014)	N/A	(Yadav et al., 2011)	(Ma et al., 2009)
AG490	EGFR JAK2	(Colomiere et al., 2009; Liu et al., 2014; Lo et al., 2007; Verma et al., 2015)	N/A	(Lu et al., 2003; Yadav et al., 2011)	(Lin et al., 2011; Ma et al., 2009; Pruvot et al., 2014)
U0126	MEK1/2	(Buonato and Lazzara, 2014; Kong et al., 2014; Latifi et al., 2011; Wan et al., 2008; Xie et al., 2004; Zheng et al., 2013)	N/A	(Amatangelo et al., 2012; Grotegut et al., 2006; Hong et al., 2011; Marchetti et al., 2008; Yu et al., 2002)	(Banjo et al., 2013; Duong et al., 2014; Hawkins et al., 2008; Ishizaki et al., 2010; Liu et al., 2008)
XL880	c-MET VEGFR2	N/A	N/A	(Lu et al., 2012; Samame Perez-Vargas et al., 2013; Silva et al., 2011)	N/A
ROCKout	ROCK	N/A	(Berndt et al., 2008; Clay and Halloran, 2013)	(Ozdamar et al., 2005; Shen et al., 2001; Tavares et al., 2006; Vardouli et al., 2005)	(Berndt et al., 2008; Clay and Halloran, 2013; Ernst et al., 2012; Harding and Nechiporuk, 2012; Weiser et al., 2007)
PD173074	FGFR	(Nguyen et al., 2013; Qian et al., 2014)	N/A	(Billottet et al., 2008; Savagner et al., 1997; Sun et al., 1999; Valles et al., 1990; Valles et al., 1996)	(Erickson et al., 2010; Letamendia et al., 2012; Luesch et al., 2006; Martinez-Morales et al., 2011)
SU5402	FGFR	(Pennisi and Mikawa, 2009; Ronca et al., 2013)	(Delfini et al., 2009; Hardy et al., 2011)	" "	(Erickson et al., 2010; Harding and Nechiporuk, 2012; Luesch et al., 2006; Molina et al., 2007)
MK2206	AKT1/2/3	N/A	N/A	(Grille et al., 2003; Gulhati et al., 2011; Irie et al., 2005; Lamouille et al., 2012; Lamouille and Derynck, 2007)	(Blackburn et al., 2014; Dai et al., 2014)
VX680	AURK A/B/C	(Wan et al., 2008)	N/A	(Chou et al., 2013; D'Assoro et al., 2014)	N/A
MLN8237	AURKA	(D'Assoro et al., 2014; Niu et al., 2015)	N/A	" "	N/A
SGI7079	AXL	(Byers et al., 2013)	N/A	(Cichon et al., 2014; Gjerdrum et al., 2010; Koorstra et al., 2009; Reichl et al., 2015; Vuoriluoto et al., 2011; Zhang et al., 2012)	N/A
MP470	AXL	(Asiedu et al., 2014)	N/A	" "	N/A
R428	AXL	(Holland et al., 2010)	N/A	" "	N/A

CHAPTER 3

ESTABLISHING AN IN VIVO ZEBRAFISH MODEL OF AXL-DEPENDENT MELANOMA INVASION

This research is in preparation for submission to a peer-reviewed journal. Laura Jimenez, Heather King, Lenard Zon, Rodney Stewart. Development of a Zebrafish Model of Axl Dependent Melanoma Invasion.

Abstract

Malignant melanoma is a highly aggressive and treatment resistant cancer. Recent advances in targeted therapies and immunotherapies developed to treat advanced melanoma are promising but fail to show significant efficacy and indicate a need to identify other therapeutic options. AXL is a receptor tyrosine kinase (RTK) that has emerged as a potential therapeutic target for melanoma. AXL is overexpressed and highly active in aggressive and undifferentiated human melanomas and its expression in melanoma cell lines regulates invasive and migratory behavior *in vitro.* Expression of AXL is upregulated in melanoma cells that acquire resistance to BRAF inhibitors, suggesting dual targeting of AXL and BRAF could lead to more durable responses in BRAF-mutant melanoma patients. Here we establish a preclinical model of AXL-dependent melanoma invasion and show for the first time *in vivo* that AXL cooperates with BRAF to transform primary melanocytes and accelerate melanoma formation, growth, and invasion. This preclinical model will aid in the identification of AXL-dependent mechanisms driving malignant melanoma and the evaluation and identification of effective AXL inhibitors for the treatment of melanoma.

Introduction

Metastatic melanoma is a notoriously aggressive and treatment-resistant disease. Therefore there is an urgent need to discover new therapies (Niezgoda et al., 2015). Recent breakthroughs have provided novel immunotherapies

targeted at PD-1 (nivolumab and pembrolizumaband) and CTLA-4 (ipilimumab) for the treatment of advanced melanoma (Dummer et al., 2015). Additionally, rational approaches, based on identifying novel melanoma target genes have shown great promise. For example, BRAF-mutant melanomas show a dramatic response in tumor regression when treated with BRAF and MEK inhibitors such as dabrafenib and trametinib, respectively (Flaherty et al., 2012a; Flaherty et al., 2012b; Mandal et al., 2015). Unfortunately, only small subsets of patients respond to immunotherapies and a majority of mutant BRAF tumors acquire resistance, resulting in more aggressive tumors that quickly overcome the patient (Strickland et al., 2015). In contrast to BRAF-mutant melanomas, NRAS-mutant tumors have very limited treatment options and poor overall survival (Ji et al., 2012; Johnson and Puzanov, 2015). Thus, new therapeutic options, validated in preclinical animal models, are needed to improve the outcomes of all melanoma patients.

The oncogenic signaling pathways that drive melanoma cooperate with genetic embryonic signals during tumor initiation and progression. The transcriptional program that drives epithelial-to-mesenchymal transition (EMT) during melanocyte development and specification has been shown to predispose melanocytes to high metastatic propensity (Gupta et al., 2005). EMT stimulates epithelial cells to dissociate from neighboring cells, which facilitates metastatic spread (Thiery et al., 2009; Yang and Weinberg, 2008). Expression of genes that induce EMT transforms melanocytes into malignant melanomas and correlates with poor patient prognosis (Alonso et al., 2007; Gupta et al., 2005; Hoek et al.,

2004). Cancer cells undergoing EMT acquire stem cell properties (Mani et al., 2008), enhanced survival (Barrallo-Gimeno and Nieto, 2005; Lu et al., 2014), escape from immune surveillance (Knutson et al., 2006; Kudo-Saito et al., 2009), and are resistance to standard chemotherapies and tyrosine kinase inhibitors (TKI) (Chung et al., 2011; Rho et al., 2009; Sequist et al., 2011). Despite numerous studies demonstrating EMT programs can contribute to many stages of cancer progression, the role of EMT in cancer metastasis is still highly debated (Tarin et al., 2005; Thompson et al., 2005). This is in part due to the fact that the EMT program has not been clearly defined during cancer progression, which makes it difficult to study in the clinic. Elucidating the pathways controlling EMT in physiologically relevant preclinical cancer models will be critical to understand the role of EMT during tumor metastasis. Additionally these studies will aid in understanding the metastatic process of cancers like melanoma and identifying therapies against the mesenchymal phenotype.

Expression of AXL receptor tyrosine kinase is strongly associated with the mesenchymal phenotype and has emerged as a potential therapeutic target. Overexpression of AXL correlates with poor patient survival in several cancers, facilitates EMT, promotes cancer stem cells, and underlies resistance to chemotherapies and TKIs (Byers et al., 2013; Dufies et al., 2011; Gjerdrum et al., 2010; Koorstra et al., 2009; Liu et al., 2009; Wilson et al., 2014; Zhang et al., 2012). AXL is highly active and overexpressed in malignant melanomas and its expression in melanoma cell lines regulates invasive and migratory behavior *in vitro* (Sensi et al., 2011; Tworkoski et al., 2011). Melanoma cells with elevated

expression of AXL acquire resistance to the PLX4720 BRAF inhibitor, suggesting that combination targeting of both BRAF and AXL may prevent BRAF resistance in patients (Johannessen et al., 2010; Villanueva et al., 2010).

Here we explore the physiological function of AXL in promoting melanoma formation and malignant progression. We established an *in vivo* zebrafish melanoma model that overexpresses AXL and tested the ability of AXL to promote melanocyte transformation and accelerate tumor initiation, growth, and invasion. We found that AXL cooperates with mutant BRAF to accelerate melanocyte transformation, tumor onset, growth, and invasion. Additionally we tested the ability of AXL to activate EMT programs *in vivo* by characterizing the signaling pathways in AXL-driven melanomas using RNA-Seq. We found AXL-overexpressing tumors negatively correlated with an EMT gene signature *in vivo* despite numerous studies demonstrating AXL can promote EMT in cancer cell lines. Additionally, we generated an AXL mutant to determine if endogenous *axl* is required to promote melanoma in our zebrafish melanoma model. These studies validate AXL as a potential therapeutic target for melanoma treatment and provide a preclinical model to explore AXL-dependent mechanisms driving neoplastic transformation, invasion, and resistance to mutant BRAF inhibitors.

Results

AXL cooperates with BRAF to promote and accelerate melanoma formation

To determine if AXL expression is sufficient to promote and accelerate melanoma *in vivo*, we generated melanoma models using transgenic zebrafish that specifically express human AXL alone or in combination with the common melanoma-associated $BRAF^{V600E}$ mutation. For our studies we used an established zebrafish melanoma model expressing $BRAF^{V600E}$ in *p53*-mutant melanocytes also lacking *mitfa* and pigment (Ceol et al., 2011). *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$*;mitfa*$^{-/-}$ fish have no melanocytes due to mutation in the transcription factor *mitfa*, thus without melanocytes these fish do not develop melanoma. However, when embryos from these fish are injected with a rescuing DNA plasmid containing the wild-type *mitfa* gene (called miniCoopR), it rescues melanocytes in a mosaic fashion and fish develop melanomas within 8-10 weeks (Ceol et al., 2011). We have engineered the miniCoopR rescue plasmid to co-express AXL and *mitfa* in the rescued melanocytes (Figure 3.1A). We cloned human AXL into the miniCoopR plasmid with a green fluorescent protein (GFP) tag and injected the rescue plasmid into *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$*;mitfa*$^{-/-}$ and p53$^{-/-}$;mitfa$^{-/-}$ embryos. Injected embryos were screened weekly for primary tumor onset followed by florescence microscopy to validate that tumors express GFP, indicative of tumors that have arisen from AXL-expressing cells (Figure 3.1A). Tumor onset was compared in parallel to embryos injected with a control rescue plasmid miniCoopR-GFP and a

positive control rescue plasmid containing SETDB1, a previously described gene known to accelerate melanoma onset (Ceol et al., 2011).

Tumor incidence curves for *p53*$^{-/-}$*;mitfa*$^{-/-}$ fish injected with miniCoopR-AXL-GFP rescue plasmid showed that AXL alone does not drive melanoma formation (Figure 3.1B). However, *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$*;mitfa*$^{-/-}$ injected with miniCoopR-AXL-GFP or miniCoopR-AXL-polyA plasmid resulted in the formation of melanoma at a significantly accelerated rate compared to *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$*;mitfa*$^{-/-}$ fish injected with control miniCoopR-GFP rescue plasmid *(p* value < .0001, Figure 3.1B). Additionally, AXL accelerates onset of melanoma similar to SETDB1, a previously reported accelerator of melanoma. These results indicate that expression of AXL alone is not sufficient to drive melanoma formation; however this is the first report demonstrating that AXL cooperates with BRAF to promote and accelerate melanoma formation.

AXL drives melanoma formation more effectively than developmental EMT programs

We compared the ability of AXL to promote tumor onset to regulators of EMT that promote malignant transformation and drive metastasis. A zebrafish melanoma model was generated overexpressing a member of the Snail family, *snai1b*, one the most widely studied families of EMT effectors. Expression of Snail family members are elevated in transformed melanocytes and enhance developmental EMT programs that promote tumor progression, invasion, and metastasis (Gupta et al., 2005; Peinado et al., 2007). As expected, we observed

snai1b overexpression significantly accelerated tumor formation compared to control tumors (Figure 3.1B). Surprisingly, AXL accelerated melanoma formation at a higher rate compared to *snai1b*-expressing melanomas. These observations indicate that AXL can promote transformation of melanocytes more effectively than drivers of developmental EMT programs, which have been shown to promote melanoma formation.

AXL transforms primary melanocytes and accelerates nevi formation

EMT can give rise to cancer stem cells and promote tumor initiation (Mani et al., 2008). In melanoma, expression of EMT effectors such as *snai1b* in nevi can malignantly transform cells and promote melanoma formation, invasion and metastasis (Alonso et al., 2007; Gupta et al., 2005; Hoek et al., 2004). Recent studies report that AXL is sufficient to directly induce EMT and activate the cancer stem cell phenotype (Asiedu et al., 2014), but the role of AXL in driving melanocyte transformation has not been explored. AXL is expressed at low levels in primary melanocytes, but is elevated in melanomas (Quong et al., 1994; Sensi et al., 2011). These observations, together with our findings that AXL overexpression can accelerate tumor onset, suggest that overexpression of AXL may promote melanocyte transformation.

To distinguish the ability of AXL to reprogram primary melanocytes and drive malignant transformation, we examined the rate of fish nevi formation in AXL-overexpressing melanocytes within our transgenic zebrafish melanoma

model. Additionally, we monitored nevi-expressing, fluorescently-tagged AXL every 2 weeks to visualize development of GFP-expressing tumor masses arising from malignantly transformed AXL-expressing cells. In our screen, fish nevi were identified as hyperpigmented patches of ectopic melanocytes at least 2 mm wide. We found that overexpression of AXL in primary melanocytes accelerated nevi formation compared to rescued melanocytes from control fish without AXL expression (Figure 3.2A). Moreover, AXL expression transformed nevi into tumor masses (Figure 3.2B). Theses studies highlight the ability of AXL to reprogram primary melanocytes and accelerate the transformation of nevi into malignant melanoma.

AXL accelerates tumor growth and invasion in vivo

Previous studies demonstrated that AXL can accelerate growth and invasion of cancer cell lines *in vitro* and promote tumor growth in mouse xenograft models (Li et al., 2009; Shieh et al., 2005; Ye et al., 2010). To determine if AXL can increase tumor growth and invasion of melanoma *in vivo* we evaluated the growth of our AXL overexpressing melanomas compared to control melanomas without AXL overexpression. Tumor size was measured with calipers once a week after tumors became visually apparent. Analysis of the tumor growth curve indicated overexpression of AXL accelerated melanoma growth *in vivo* compared to control tumors (Figure 3.3A). Additionally melanomas with AXL overexpression were more aggressive than control tumors generated in the same *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$*;mitfa*$^{-/-}$ genetic background. Histological

sections of tumors revealed AXL-expressing melanomas become more invasive at early stages of tumor development. Melanomas from control tumors assessed at a later stage of tumor progression, 5 mm^3, grow outward on the skin and do not break though the basement membrane (Figure 3.3B). This is in contrast to AXL-driven melanomas that begin to locally invade and break through the basement membrane by 1 mm^3 (Figure 3.3B). As the AXL-driven melanomas continue to develop and reach 3 mm^3 in size, they completely invade the underlying muscle. These studies show for the first time that AXL can enhance melanoma growth and invasion *in vivo*.

AXL-dependent signaling pathways driving melanoma onset and invasion

To determine which AXL-dependent signaling pathways regulate melanoma onset and invasion, we performed RNA-Seq analysis on AXL-overexpressing and control tumors. We collected tumors within the same size range of approximately 2 mm^3, an earlier stage of tumor development, when differences in transcriptional invasion programs can be clearly assessed by RNA-Seq analysis. RNA was extracted from AXL-overexpressing tumors and control tumors to analyze relative levels of gene expression. We hypothesized AXL expression would activate EMT programs to drive melanoma invasion. Surprisingly, through a gene-set-enrichment analysis (GSEA) we identified the EMT pathway was significantly downregulated in AXL-overexpressing tumors (Figure 3.4A). These results indicate that AXL may function through an

alternative method *in vivo*. Interestingly, we found a significant enrichment for factors involved in the blood coagulation system, which could represents a novel AXL-dependent mechanism driving transformation and invasion of cancer cells.

Future direction: is AXL required to promote melanoma onset, growth, and invasion?

To directly test whether AXL is required for accelerated tumor onset, growth, and invasion we aimed to generate a zebrafish melanoma model lacking endogenous zebrafish *axl* expression. To test this, we generated a zebrafish *axl* mutant to cross into the *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$*;mitfa*$^{-/-}$ transgenic zebrafish melanoma model. To generate an *axl* mutant we used the CRISPR/Cas9 system, a new genome editing technology validated in zebrafish (Chang et al., 2013; Hruscha et al., 2013; Jao et al., 2013). With help from the University of Utah mutagenesis core we generated a guide RNA (gRNA) designed to target exon 4 of the *axl* locus and several null alleles of ax*l* were generated. The *axl*$^{\Delta 4}$*, axl*$^{\Delta 7}$*, axl*$^{\Delta 11}$ alleles all harbor frame-shift deletions in exon 4 that generate premature stop codons upstream of the transmembrane domain and the kinase domain that is required for AXL signaling (Braunger et al., 1997; Fridell et al., 1996).

To generate zebrafish melanomas lacking endogenous *axl* expression, the *axl* mutant will be generated in the *p53*$^{-/-}$*;mitfa*$^{-/-}$ genetic background and crossed to the *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$*;mitfa*$^{-/-}$ melanoma model. MiniCoopR-MITF-RFP rescue plasmids will be injected into 1-cell stage

Tg(mitfa:BRAF(V600E));p53$^{-/-}$*;mitfa*$^{-/-}$ and *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$*;mitfa*$^{-/-}$*;axl*$^{-/-}$ zebrafish embryos and tumor onset, growth, and invasion will be compared in the two genetic backgrounds (Figure 3.5A). AXL has previously been implicated in regulating tumor stroma through modulation of tumor-associated vasculature and immune cell function (Ye et al., 2010). To test whether *axl* is necessary in the tumor microenvironment to promote melanoma invasion, miniCoopR-AXL-GFP will be injected into the *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$*;mitfa*$^{-/-}$ and *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$*;mitfa*$^{-/-}$*;axl*$^{-/-}$ genetic backgrounds and tumor onset growth and invasion will be compared (Figure 3.5B). Together these studies will determine the necessity of *axl* in regulating multiple roles of tumorgenesis.

Discussion

In this study, we investigated the various roles of AXL in melanoma, including its role in promoting melanocyte transformation, tumor initiation/onset, growth, and invasion. We generated an *in vivo* zebrafish model of AXL-driven melanoma and demonstrated that AXL cooperates with the BRAF^{V600E} mutation to accelerate melanoma onset. Additionally, we found that cooperation of AXL with BRAF^{V600E} mutation accelerated melanocyte transformation into nevi and promoted tumor growth and invasion *in vivo*. Finally, our RNA sequencing analysis of AXL-overexpressing tumors revealed AXL does not activate EMT programs *in vivo* as previously suggested from *in vitro* studies and indicates other AXL dependent pathways may be regulating invasion in our zebrafish

melanoma model. These studies provide the first evidence that AXL can cooperate with BRAF mutations to promote melanoma *in vivo* and support the development of targeted therapy against AXL for the treatment of BRAF-driven melanomas.

Melanoma is the most aggressive form of skin cancer, notorious for its resistance to chemotherapy and highly metastatic nature in the clinic. Over 50% of melanoma patients harboring $BRAF^{v600E}$-activating mutations benefit from targeted therapies against BRAF (Chapman et al., 2011; Curtin et al., 2005; Davies et al., 2002). However, most patients who initially respond to BRAF inhibitors acquire drug resistance and relapse (Flaherty, 2011). Understanding the mechanisms by which tumors acquire resistance may lead to development of treatment strategies with enhanced clinical efficacy. Activation of AXL in breast and lung cancer cell lines can promote resistance to targeted therapies and provides new mechanisms of resistance to explore for melanoma patients (Liu et al., 2009; Zhang et al., 2012). Interestingly, upregulation of AXL occurs during acquired resistance to the PLX4720 BRAF inhibitor and expression of AXL has been observed in 30% of BRAF-mutant melanomas (Johannessen et al., 2010; Sensi et al., 2011; Villanueva et al., 2010). These observations suggest that combined targeting of both BRAF and AXL may prevent BRAF resistance in patients, or at least prolong the effects of current BRAF inhibitors. Indeed, treatment of human melanoma cells with AXL inhibitors *in vitro* decreased proliferation, migration, and invasion, supporting the notion that targeting AXL in combination with other proteins may have significant impact on patients with

metastatic melanoma (Sensi et al., 2011; Tworkoski et al., 2011). Additionally, expression of AXL is significantly more frequent in NRAS-mutated melanomas and suggests that AXL inhibitors may also selectively kill this subtype of melanoma. Our study provides additional support for the combinatorial use of AXL and BRAF inhibitors to treat melanoma patients and warrants further investigation to assess whether AXL underlies resistance to BRAF inhibitors. Future studies using our preclinical model of AXL and BRAF-driven melanoma will help distinguish the role of AXL in regulating resistance to mutant BRAF inhibitors.

The presence of cancer stem cells limits the response to anti-cancer treatments such as BRAF inhibitors. Recent studies have highlighted a link between EMT and the formation of cancer stem cells (Mani et al., 2008). In cancer cells, induction of EMT can generate cancer stem cells capable of self-renewal, tumor initiation and acquired resistance to therapies. AXL exemplifies one signaling pathway that can be exploited in cancer cells known to induce EMT, regulate self renewal, and promote chemoresistance of cancer stem cells *in vitro* (Asiedu et al., 2014; Byers et al., 2013; Gjerdrum et al., 2010; Wilson et al., 2014). In human melanoma, expression of AXL is correlated with poorly differentiated tumors that have increased metastatic potential and resistance to treatment (Hoek et al., 2006). In our studies, overexpression of AXL accelerates melanoma onset more effectively than inducers of EMT such as *snai1b* (Caramel et al., 2013). However, tumors arising from malignantly transformed AXL-expressing cells negatively correlated with an EMT gene signature. Results from

our RNA-Seq data suggest that the high degree of cellular plasticity and invasion acquired in AXL-overexpressing cancer cells is not mediated through EMT programs as previously suggested and indicates the physiological function of AXL may work though an alternative method. Interestingly the gene-set-enrichment analysis of our RNA-Seq data identified a significant enrichment for factors involved in the blood coagulation system. Very little is known about the role of coagulation in cancer progression but studies suggest it can promote tumor cell spreading (Im et al., 2004) and anticoagulants can suppress cancer metastasis in experimental models and cancer patients (Gil-Bernabe et al., 2013). Further studies are necessary to validate whether AXL is inducing coagulation to promote invasion in our zebrafish melanoma model. Nevertheless, these results indicate AXL may be part of a more effective therapy to target invasive and resistant cancer cells.

Methods

Generation of zebrafish melanoma models

MiniCoopR plasmids was obtained from (Ceol et al., 2011) and individual clones were created by MultiSite Gateway recombination (Invitrogen) using full-length open reading frames. Twenty-five pictograms of each miniCoopR clone and 25 pg mRNA encoding theTol2 transposase were microinjected into one-cell zebrafish embryos. Embryos were generated from an incross of *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$*;mitfa*$^{-/-}$ and *p53*$^{-/-}$*;mitfa*$^{-/-}$ zebrafish. Injected animals with rescued melanocytes were grown up to adulthood and screened weekly for

the presence of nevi or visible tumors staring at 6 weeks of age.

Measuring zebrafish tumor growth

Tumor volume was determined *in vivo* with caliper. Five fish for each tumor type were monitored weekly to asses tumor growth. Tumors were measured as soon as the tumor became visible. A caliper was used to measure the length and width of the tumor and volume was calculated using a modified ellipsoid formula ½(Length x width^2).

Histological analysis of zebrafish tumors to analyze invasion

Zebrafish with tumors were fixed overnight in 4% PFA and embedded and sectioned in paraffin. Sections were stained with hematoxylin and eosin.

RNA sequencing of zebrafish tumors

Total RNA was harvested from three miniCoopR-AXL-GFP melanomas and three miniCoopR-MITF-GFP melanomas using Qiagen RNeasy kit (Cat # 74104). Quality of RNA was assessed using Bioanalyzer RNA 6000 Nano Chip and RNA-Seq libraries prepared using illumina TruSeq Stranded Total RNA Sample Prep Kit with Ribo-Zero (Cat # RS-122-2401). Sequencing was performed on Illumina HiSeq 2000 using 50 cycle single-read sequencing v3 kit (Cat # FC-401-3002). Illumina Fatq files were aligned using Novoalign to the zebrafish Zv9 genome. All high-throughput sequencing data sets will be submitted to Gene Expression Omnibus (GEO).

References

Alonso, S. R., Tracey, L., Ortiz, P., Perez-Gomez, B., Palacios, J., Pollan, M., Linares, J., Serrano, S., Saez-Castillo, A. I., Sanchez, L. et al. (2007). A high-throughput study in melanoma identifies epithelial-mesenchymal transition as a major determinant of metastasis. *Cancer Res* **67**, 3450-60.

Asiedu, M. K., Beauchamp-Perez, F. D., Ingle, J. N., Behrens, M. D., Radisky, D. C. and Knutson, K. L. (2014). AXL induces epithelial-to-mesenchymal transition and regulates the function of breast cancer stem cells. *Oncogene* **33**, 1316-24.

Barrallo-Gimeno, A. and Nieto, M. A. (2005). The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* **132**, 3151-61.

Braunger, J., Schleithoff, L., Schulz, A. S., Kessler, H., Lammers, R., Ullrich, A., Bartram, C. R. and Janssen, J. W. (1997). Intracellular signaling of the Ufo/Axl receptor tyrosine kinase is mediated mainly by a multi-substrate docking-site. *Oncogene* **14**, 2619-31.

Byers, L. A., Diao, L., Wang, J., Saintigny, P., Girard, L., Peyton, M., Shen, L., Fan, Y., Giri, U., Tumula, P. K. et al. (2013). An epithelial-mesenchymal transition gene signature predicts resistance to EGFR and PI3K inhibitors and identifies Axl as a therapeutic target for overcoming EGFR inhibitor resistance. *Clin Cancer Res* **19**, 279-90.

Caramel, J., Papadogeorgakis, E., Hill, L., Browne, G. J., Richard, G., Wierinckx, A., Saldanha, G., Osborne, J., Hutchinson, P., Tse, G. et al. (2013). A switch in the expression of embryonic EMT-inducers drives the development of malignant melanoma. *Cancer Cell* **24**, 466-80.

Ceol, C. J., Houvras, Y., Jane-Valbuena, J., Bilodeau, S., Orlando, D. A., Battisti, V., Fritsch, L., Lin, W. M., Hollmann, T. J., Ferre, F. et al. (2011). The histone methyltransferase SETDB1 is recurrently amplified in melanoma and accelerates its onset. *Nature* **471**, 513-7.

Chang, N., Sun, C., Gao, L., Zhu, D., Xu, X., Zhu, X., Xiong, J. W. and Xi, J. J. (2013). Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. *Cell Res* **23**, 465-72.

Chapman, P. B., Hauschild, A., Robert, C., Haanen, J. B., Ascierto, P., Larkin, J., Dummer, R., Garbe, C., Testori, A., Maio, M. et al. (2011). Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* **364**, 2507-16.

Chung, J. H., Rho, J. K., Xu, X., Lee, J. S., Yoon, H. I., Lee, C. T., Choi, Y. J., Kim, H. R., Kim, C. H. and Lee, J. C. (2011). Clinical and molecular evidences of epithelial to mesenchymal transition in acquired resistance to EGFR-TKIs. *Lung Cancer* **73**, 176-82.

Curtin, J. A., Fridlyand, J., Kageshita, T., Patel, H. N., Busam, K. J., Kutzner, H., Cho, K. H., Aiba, S., Brocker, E. B., LeBoit, P. E. et al. (2005). Distinct sets of genetic alterations in melanoma. *N Engl J Med* **353**, 2135-47.

Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W. et al. (2002). Mutations of the BRAF gene in human cancer. *Nature* **417**, 949-54.

Dufies, M., Jacquel, A., Belhacene, N., Robert, G., Cluzeau, T., Luciano, F., Cassuto, J. P., Raynaud, S. and Auberger, P. (2011). Mechanisms of AXL overexpression and function in Imatinib-resistant chronic myeloid leukemia cells. *Oncotarget* **2**, 874-85.

Dummer, R., Schadendorf, D., Ascierto, P. A., Larkin, J., Lebbe, C. and Hauschild, A. (2015). Integrating first-line treatment options into clinical practice: what's new in advanced melanoma? *Melanoma Research* **25**, 461-469.

Flaherty, K. T. (2011). BRAF inhibitors and melanoma. *Cancer J* **17**, 505-11.

Flaherty, K. T., Infante, J. R., Daud, A., Gonzalez, R., Kefford, R. F., Sosman, J., Hamid, O., Schuchter, L., Cebon, J., Ibrahim, N. et al. (2012a). Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med* **367**, 1694-703.

Flaherty, K. T., Robert, C., Hersey, P., Nathan, P., Garbe, C., Milhem, M., Demidov, L. V., Hassel, J. C., Rutkowski, P., Mohr, P. et al. (2012b). Improved survival with MEK inhibition in BRAF-mutated melanoma. *N Engl J Med* **367**, 107-14.

Fridell, Y. W., Jin, Y., Quilliam, L. A., Burchert, A., McCloskey, P., Spizz, G., Varnum, B., Der, C. and Liu, E. T. (1996). Differential activation of the Ras/extracellular-signal-regulated protein kinase pathway is responsible for the biological consequences induced by the Axl receptor tyrosine kinase. *Mol Cell Biol* **16**, 135-45.

Gil-Bernabe, A. M., Lucotti, S. and Muschel, R. J. (2013). Coagulation and metastasis: what does the experimental literature tell us? *Br J Haematol* **162**, 433-41.

Gjerdrum, C., Tiron, C., Hoiby, T., Stefansson, I., Haugen, H., Sandal,

T., Collett, K., Li, S., McCormack, E., Gjertsen, B. T. et al. (2010). Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival. *Proc Natl Acad Sci U S A* **107**, 1124-9.

Gupta, P. B., Kuperwasser, C., Brunet, J. P., Ramaswamy, S., Kuo, W. L., Gray, J. W., Naber, S. P. and Weinberg, R. A. (2005). The melanocyte differentiation program predisposes to metastasis after neoplastic transformation. *Nat Genet* **37**, 1047-54.

Hoek, K., Rimm, D. L., Williams, K. R., Zhao, H., Ariyan, S., Lin, A., Kluger, H. M., Berger, A. J., Cheng, E., Trombetta, E. S. et al. (2004). Expression profiling reveals novel pathways in the transformation of melanocytes to melanomas. *Cancer Res* **64**, 5270-82.

Hoek, K. S., Schlegel, N. C., Brafford, P., Sucker, A., Ugurel, S., Kumar, R., Weber, B. L., Nathanson, K. L., Phillips, D. J., Herlyn, M. et al. (2006). Metastatic potential of melanomas defined by specific gene expression profiles with no BRAF signature. *Pigment Cell Res* **19**, 290-302.

Hruscha, A., Krawitz, P., Rechenberg, A., Heinrich, V., Hecht, J., Haass, C. and Schmid, B. (2013). Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. *Development* **140**, 4982-7.

Im, J. H., Fu, W., Wang, H., Bhatia, S. K., Hammer, D. A., Kowalska, M. A. and Muschel, R. J. (2004). Coagulation facilitates tumor cell spreading in the pulmonary vasculature during early metastatic colony formation. *Cancer Res* **64**, 8613-9.

Jao, L. E., Wente, S. R. and Chen, W. (2013). Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc Natl Acad Sci U S A* **110**, 13904-9.

Ji, Z., Flaherty, K. T. and Tsao, H. (2012). Targeting the RAS pathway in melanoma. *Trends Mol Med* **18**, 27-35.

Johannessen, C. M., Boehm, J. S., Kim, S. Y., Thomas, S. R., Wardwell, L., Johnson, L. A., Emery, C. M., Stransky, N., Cogdill, A. P., Barretina, J. et al. (2010). COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature* **468**, 968-72.

Johnson, D. B. and Puzanov, I. (2015). Treatment of NRAS-mutant melanoma. *Curr Treat Options Oncol* **16**, 15.

Knutson, K. L., Lu, H., Stone, B., Reiman, J. M., Behrens, M. D., Prosperi, C. M., Gad, E. A., Smorlesi, A. and Disis, M. L. (2006). Immunoediting of cancers may lead to epithelial to mesenchymal transition. *J*

Immunol **177**, 1526-33.

Koorstra, J. B., Karikari, C. A., Feldmann, G., Bisht, S., Rojas, P. L., Offerhaus, G. J., Alvarez, H. and Maitra, A. (2009). The Axl receptor tyrosine kinase confers an adverse prognostic influence in pancreatic cancer and represents a new therapeutic target. *Cancer Biol Ther* **8**, 618-26.

Kudo-Saito, C., Shirako, H., Takeuchi, T. and Kawakami, Y. (2009). Cancer metastasis is accelerated through immunosuppression during Snail-induced EMT of cancer cells. *Cancer Cell* **15**, 195-206.

Li, Y., Ye, X., Tan, C., Hongo, J. A., Zha, J., Liu, J., Kallop, D., Ludlam, M. J. and Pei, L. (2009). Axl as a potential therapeutic target in cancer: role of Axl in tumor growth, metastasis and angiogenesis. *Oncogene* **28**, 3442-55.

Liu, L., Greger, J., Shi, H., Liu, Y., Greshock, J., Annan, R., Halsey, W., Sathe, G. M., Martin, A. M. and Gilmer, T. M. (2009). Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL. *Cancer Res* **69**, 6871-8.

Lu, M., Marsters, S., Ye, X., Luis, E., Gonzalez, L. and Ashkenazi, A. (2014). E-cadherin couples death receptors to the cytoskeleton to regulate apoptosis. *Mol Cell* **54**, 987-98.

Mandal, R., Becker, S. and Strebhardt, K. (2015). Stamping out RAF and MEK1/2 to inhibit the ERK1/2 pathway: an emerging threat to anticancer therapy. *Oncogene* **38**, 1-15.

Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., Brooks, M., Reinhard, F., Zhang, C. C., Shipitsin, M. et al. (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133**, 704-15.

Niezgoda, A., Niezgoda, P. and Czajkowski, R. (2015). Novel Approaches to treatment of advanced melanoma: a review on targeted therapy and immunotherapy. *Biomed Res Int* **2015**, 851387.

Peinado, H., Olmeda, D. and Cano, A. (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer* **7**, 415-28.

Quong, R. Y., Bickford, S. T., Ing, Y. L., Terman, B., Herlyn, M. and Lassam, N. J. (1994). Protein kinases in normal and transformed melanocytes. *Melanoma Res* **4**, 313-9.

Rho, J. K., Choi, Y. J., Lee, J. K., Ryoo, B. Y., Na, II, Yang, S. H., Kim,

C. H. and Lee, J. C. (2009). Epithelial to mesenchymal transition derived from repeated exposure to gefitinib determines the sensitivity to EGFR inhibitors in A549, a non-small cell lung cancer cell line. *Lung Cancer* **63**, 219-26.

Sensi, M., Catani, M., Castellano, G., Nicolini, G., Alciato, F., Tragni, G., De Santis, G., Bersani, I., Avanzi, G., Tomassetti, A. et al. (2011). Human cutaneous melanomas lacking MITF and melanocyte differentiation antigens express a functional Axl receptor kinase. *J Invest Dermatol* **131**, 2448-57.

Sequist, L. V., Waltman, B. A., Dias-Santagata, D., Digumarthy, S., Turke, A. B., Fidias, P., Bergethon, K., Shaw, A. T., Gettinger, S., Cosper, A. K. et al. (2011). Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci Transl Med* **3**, 75ra26.

Shieh, Y. S., Lai, C. Y., Kao, Y. R., Shiah, S. G., Chu, Y. W., Lee, H. S. and Wu, C. W. (2005). Expression of axl in lung adenocarcinoma and correlation with tumor progression. *Neoplasia* **7**, 1058-64.

Strickland, L. R., Pal, H. C., Elmets, C. A. and Afaq, F. (2015). Targeting drivers of melanoma with synthetic small molecules and phytochemicals. *Cancer Lett* **359**, 20-35.

Tarin, D., Thompson, E. W. and Newgreen, D. F. (2005). The fallacy of epithelial mesenchymal transition in neoplasia. *Cancer Res* **65**, 5996-6000; discussion 6000-1.

Thiery, J. P., Acloque, H., Huang, R. Y. and Nieto, M. A. (2009). Epithelial-mesenchymal transitions in development and disease. *Cell* **139**, 871-90.

Thompson, E. W., Newgreen, D. F. and Tarin, D. (2005). Carcinoma invasion and metastasis: a role for epithelial-mesenchymal transition? *Cancer Res* **65**, 5991-5; discussion 5995.

Tworkoski, K., Singhal, G., Szpakowski, S., Zito, C. I., Bacchiocchi, A., Muthusamy, V., Bosenberg, M., Krauthammer, M., Halaban, R. and Stern, D. F. (2011). Phosphoproteomic screen identifies potential therapeutic targets in melanoma. *Mol Cancer Res* **9**, 801-12.

Villanueva, J., Vultur, A., Lee, J. T., Somasundaram, R., Fukunaga-Kalabis, M., Cipolla, A. K., Wubbenhorst, B., Xu, X., Gimotty, P. A., Kee, D. et al. (2010). Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. *Cancer Cell* **18**, 683-95.

Wilson, C., Ye, X., Pham, T., Lin, E., Chan, S., McNamara, E., Neve, R.

M., Belmont, L., Koeppen, H., Yauch, R. L. et al. (2014). AXL inhibition sensitizes mesenchymal cancer cells to antimitotic drugs. *Cancer Res* **74**, 5878-90.

Yang, J. and Weinberg, R. A. (2008). Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell* **14**, 818-29.

Ye, X., Li, Y., Stawicki, S., Couto, S., Eastham-Anderson, J., Kallop, D., Weimer, R., Wu, Y. and Pei, L. (2010). An anti-Axl monoclonal antibody attenuates xenograft tumor growth and enhances the effect of multiple anticancer therapies. *Oncogene* **29**, 5254-64.

Zhang, Z., Lee, J. C., Lin, L., Olivas, V., Au, V., LaFramboise, T., Abdel-Rahman, M., Wang, X., Levine, A. D., Rho, J. K. et al. (2012). Activation of the AXL kinase causes resistance to EGFR-targeted therapy in lung cancer. *Nat Genet* **44**, 852-60.

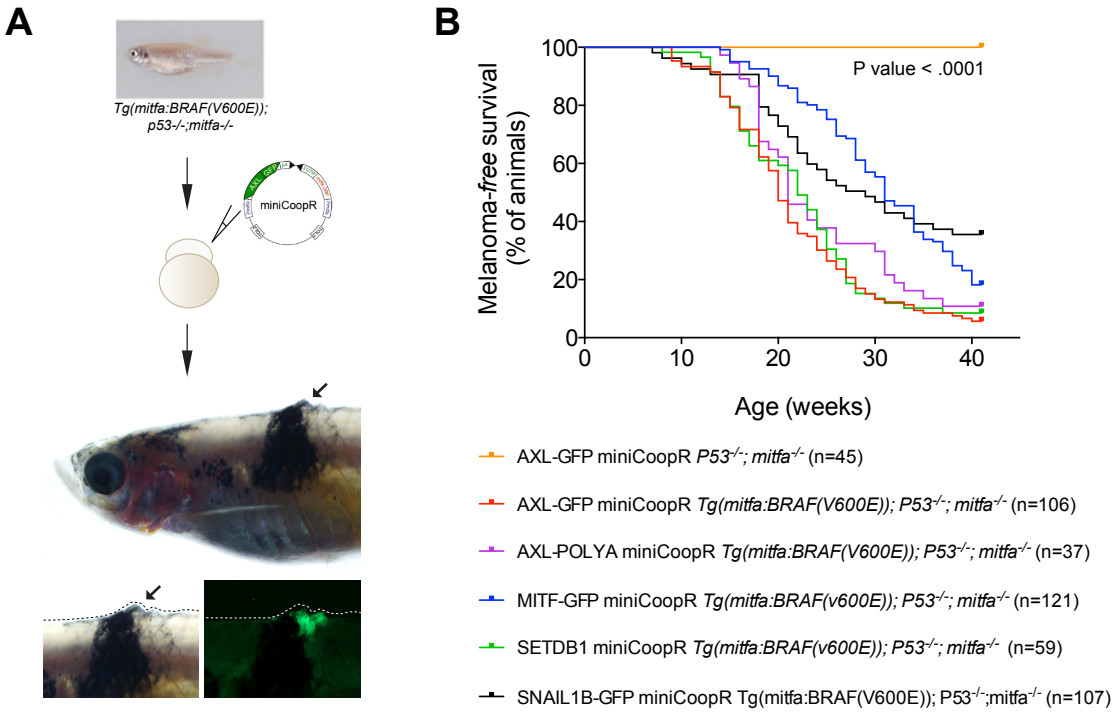


Figure 3.1. AXL cooperates with BRAF to accelerate melanoma onset. (A) Transgenic zebrafish melanoma model overexpressing fluorescently tagged AXL generated by injecting *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$*;mitfa*$^{-/-}$ transgenic zebrafish embryos at 1 cell stage with the AXL-GFP miniCoopR plasmid. Injection of embryos results in overexpression of fluorescently tagged AXL in rescued melanocytes and can be visualized in the bottom image of a developing melanoma. MiniCoopR vector image modified from (Ceol et al., 2011). (B) Melanoma-free survival curves for *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$*;mitfa*$^{-/-}$ *or p53*$^{-/-}$*;mitfa*$^{-/-}$ injected with indicated miniCoopR plasmids (total number of fish for each survival curve analysis was generated from 2 independent experiments except *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$*;mitfa*$^{-/-}$ injected with AXL-polyA miniCoopR and *p53*$^{-/-}$*;mitfa*$^{-/-}$ injected with AXL-GFP miniCoopR).

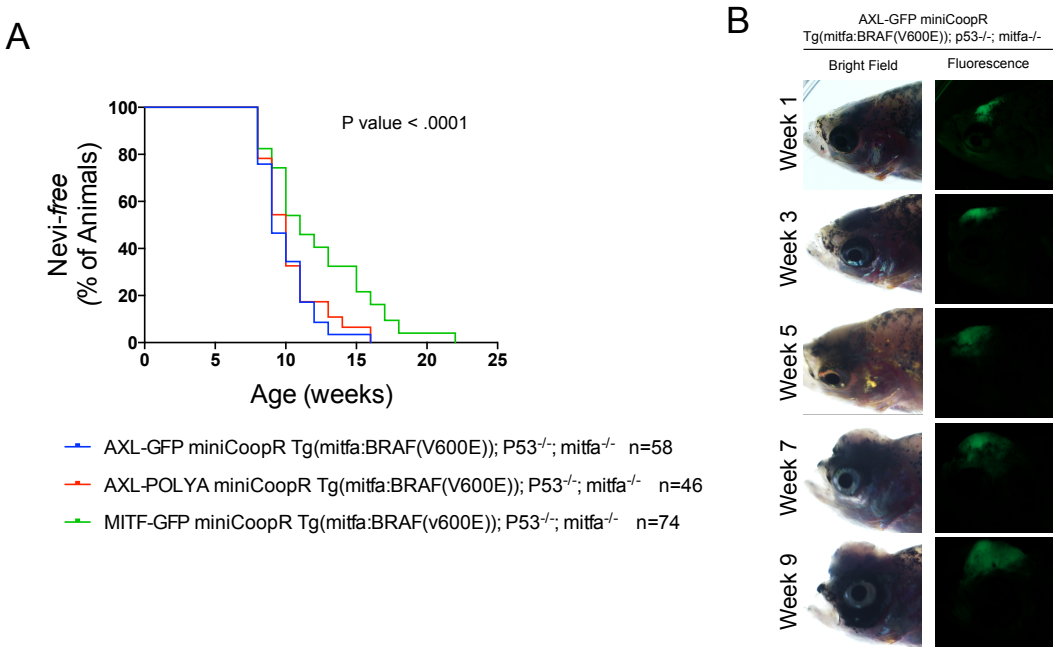
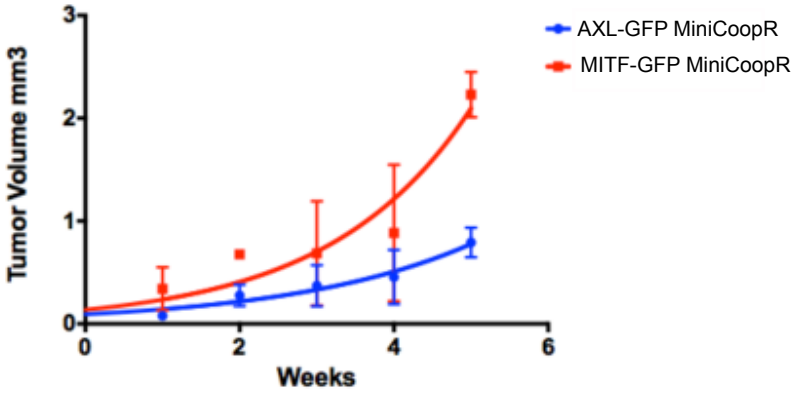


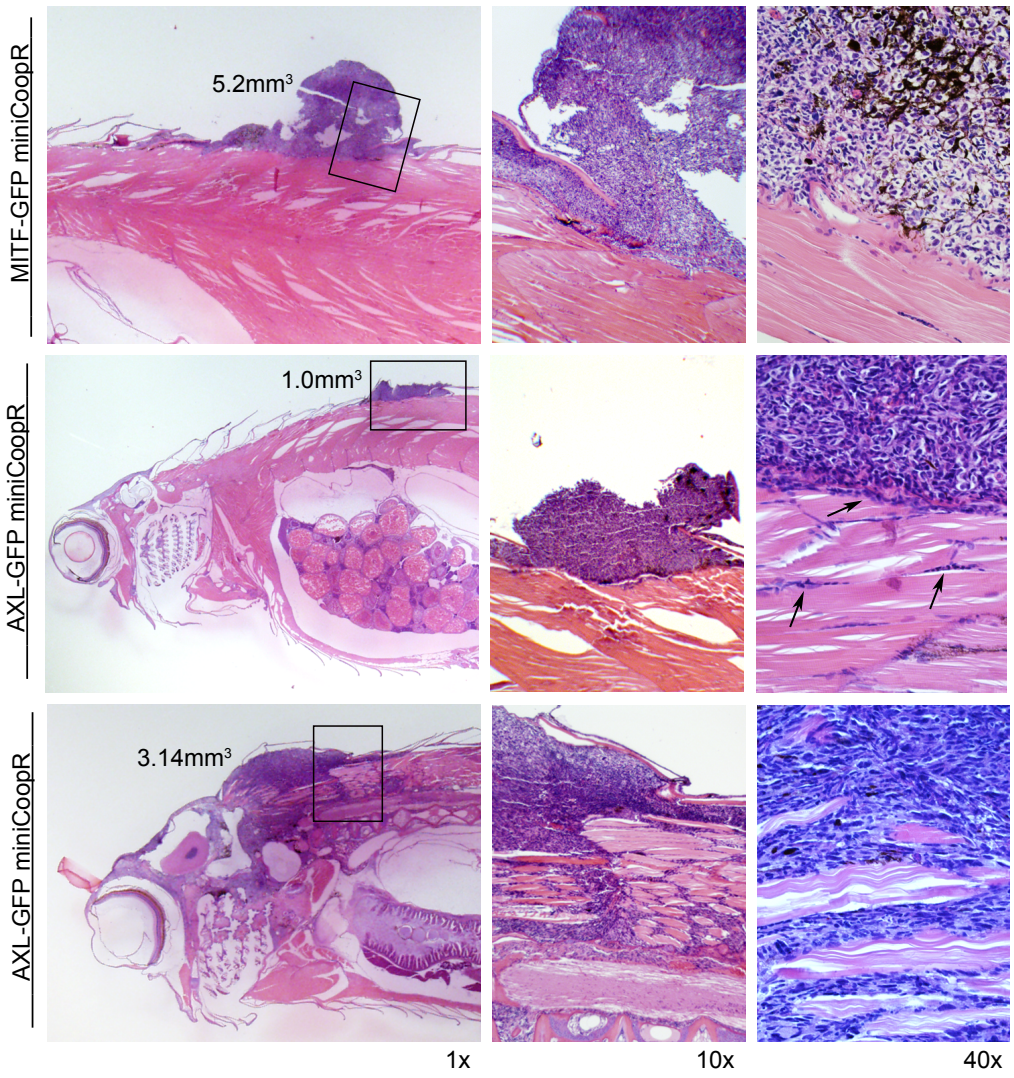
Figure 3.2. AXL cooperates with BRAF to transform primary melanocytes and promote tumor formation**.** (A) Nevi-free survival curves showing *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$*;mitfa*$^{-/-}$ injected with AXL-GFP, AXL-polyA, or MITF-GFP miniCoopR plasmid indicates overexpression of AXL in primary melanocytes accelerates nevi formation. (B) Nevi expressing fluorescently tagged AXL monitored every 2 weeks confirms GFP-expressing tumor mass arise from malignantly transformed AXL expressing cells n=6. n = number of fish.

Figure 3.3. AXL accelerates melanoma growth and enhances invasion *in vivo.* (A) Tumor volume area in AXL overexpressing melanomas compared to GFP control melanomas. (B) Sections of zebrafish melanomas at different time points of tumor progression stained with hematoxylin and eosin. At a later stage of tumor progression, 5 mm^3, control melanomas grow outwards on the skin and do not break through and invade the basement membrane. AXL overexpressing melanomas start to invade through the basement membrane (arrows) at an early stage 1 mm^3 and by 3 mm^3 they have completely invaded through the skin into the underlying muscle.

A


AXL-GFP MiniCoopR
MITF-GFP MiniCoopR
Tumor Volume mm3
Weeks
0
1
2
3
0
2
4
6

B


MITF-GFP miniCoopR
AXL-GFP miniCoopR
AXL-GFP miniCoopR
5.2mm3
1.0mm3
3.14mm3
1x
10x
40x

A

AXL-overexpressing melanoma

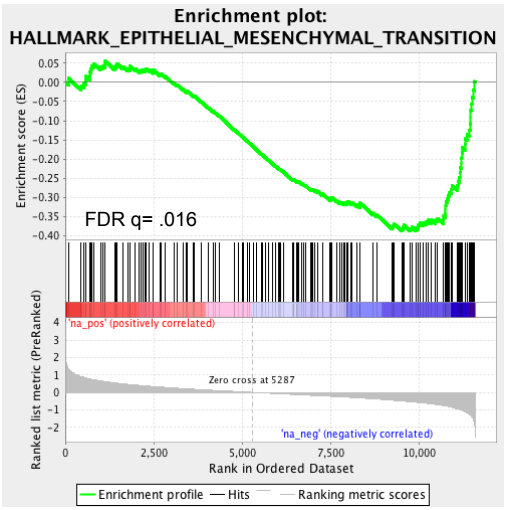


Figure 3.4. GSEA in AXL overexpressing tumors reveals negative correlation with EMT pathway. (A) Gene-set-enrichment analysis (GSEA) plots of gene expression signatures of Hallmark_Epithelial_mesenchymal_Transition. The barcode plot indicates the position of the genes in each gene set; red and blue colors represent positive and negative correlation with AXL expression. FDR, false discovery rate.

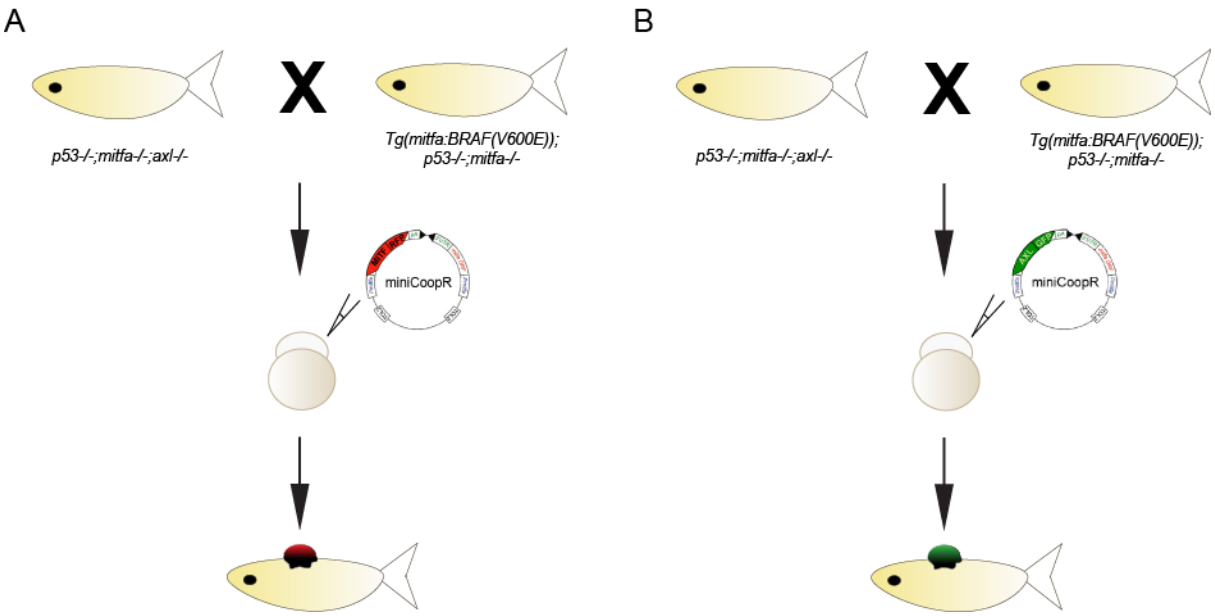


Figure 3.5. Experimental design to test the requirement of AXL in promoting melanoma onset, growth, and invasion. (A) Generation of zebrafish melanoma model lacking AXL expression will be generated by crossing *p53*$^{-/-}$;*mitfa*$^{-/-}$;*axl*$^{-/-}$ into the *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$;*mitfa*$^{-/-}$ genetic background. Embryos will be injected with control MITF-RFP miniCoopR plasmid and tumor onset, growth, and invasion will be compared to *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$;*mitfa*$^{-/-}$ injected with the same control MITF-RFP miniCoopR plasmid. (B) To test whether AXL is necessary in the tumor microenvironment to promote melanoma invasion the AXL-GFP miniCoopR plasmid will be injected into the *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$;*mitfa*$^{-/-}$;*axl*$^{-/-}$ genetic background and tumor onset, growth and invasion will be compared to *Tg(mitfa:BRAF(V600E));p53*$^{-/-}$;*mitfa*$^{-/-}$ injected with the same AXL-GFP miniCoopR plasmid.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Zebrafish as a model system for preclinical drug screening

In this body of work we apply the zebrafish model system to identify novel signaling pathways regulating EMT and cancer cell invasion *in vivo.* We took advantage of the genetic and transparent attributes of the zebrafish and generated a novel *in vivo* EMT reporter called *Tg(snai1b:GFP),* which allows direct visualization of EMT in an intact animal. This transgenic line fluorescently labels *snai1b*-expressing cells undergoing EMT during development, including dorsal neural tube progenitors undergoing EMT to form Neural Crest (NC). The *Tg(snai1b:GFP)* was used to rapidly screen small molecules and identify novel *in vivo* inhibitors of EMT. We identified a multi-kinase inhibitor TP-0903, which potently blocks EMT by increasing RA biosynthesis. We provide the first study to implicate RA as an antagonist of EMT and establish a complementary *in vivo* approach to identify effective EMT inhibitors for clinical application.

Through our chemical genetic screening approach to identify inhibitors of EMT, we gained insight into signaling pathways that contribute to invasion mechanisms and can be rationally targeted as a therapeutic strategy. Our findings of multi-kinase inhibitor TP-0903 presented AXL as a therapeutic target and a candidate for promoting cancer cell invasion *in vivo*. TP-0903 was rationally designed to target AXL RTK and this strongly suggests AXL is a candidate in promoting EMT and invasion of NC cells and NC derived cancers like melanoma. We tested the ability of AXL to accelerate cancer progression in vivo by generating a zebrafish melanoma model overexpressing AXL. We found AXL cooperates with BRAF to accelerate melanocyte transformation, tumor

onset, growth, and invasion *in vivo*. These studies provide the first evidence that AXL can cooperate with BRAF to promote melanoma *in vivo* and support the development of AXL inhibitors for the treatment of melanoma. Additionally the establishment of an *in vivo* model of AXL dependent invasion will help elucidate the AXL dependent mechanisms driving EMT, invasion, and resistance to therapies.

Together these studies demonstrate the strength of using zebrafish for preclinical drug screening and highlight zebrafish as a key model system to help identify functional regulator of cancer progression *in vivo.*

Developing AXL inhibitors for cancer therapy

Here we provide clear evidence supporting the clinical development of AXL kinase inhibitors as an antimetastatic agent. Currently there are several small molecule inhibitors being developed to target AXL, including TP-0903 that is in preclinical development by Tolero Pharmaceuticals (Tolero Pharmaceuticals, 2015). TP-0903 is a multitargeted kinase inhibitor with high potency against AXL (IC_{50} = 27 nmol/L) and significant efficacy against multiple types of cancers *in vitro* including leukemia, pancreatic, and lung cancer (Mollard et al., 2011; Sinha et al., 2015). Other multi-kinase inhibitors being actively developed with inhibitory activity against AXL include MP470 (Amuvatinib), which has been shown to synergize with docetaxel and is cytotoxic to gastrointestinal tumors and breast cancer (Mahadevan et al., 2007). Currently compound R428 (BGB324) is one of the most potent and highly selective AXL inhibitors published

and entered phase 1 clinical trial in 2013 (Sheridan, 2013). In preclinical studies R428 reduced invasion of cancer cells *in vitro* and suppressed metastasis in mouse models of metastatic breast cancer (Holland et al., 2010). Additionally, SGI-7079 is another highly specific AXL inhibitor that is currently in phase 2 clinical trials for demonstrating that it can decrease malignant properties in inflammatory breast cancer and can overcome resistance to EGFR inhibitors in NSCLC in preclinical studies (Byers et al., 2013; Wang et al., 2013). So far inhibition of AXL signaling as a cancer treatment strategy has shown great promise and multiple drug companies are pursuing the clinical development of other AXL inhibitors (Wu et al., 2014). Future studies will benefit from the zebrafish model of AXL dependent melanoma invasion developed in our studies to evaluate and identify effective AXL inhibitors *in vivo.*

Monoclonal antibodies are also being generated as a novel strategy to inhibit AXL signaling. Antibodies have been developed against the extracellular domain of AXL and the AXL ligand Gas6 (Angelillo-Scherrer et al., 2005; Ye et al., 2010). Anti-AXL monoclonal antibody YW327.6S2 has shown highly specific activity against AXL and can attenuate tumor growth and reduce metastasis in a xenograft model of breast cancer (Ye et al., 2010). The Anti-Gas6 antibody has not been investigated for the treatment of cancer but it has proven to be useful in neutralizing Gas6 activity and reducing signaling of all AXL family members (Angelillo-Scherrer et al., 2005). Moving forward with the use of AXL-targeted therapies in human cancers, the development of appropriate biomarkers will be crucial to identify a population of patients who will respond and benefit from

treatment with AXL inhibitors. Currently no genetic mutations have been associated with the oncogenic properties of AXL and using IHC to detect AXL overexpression seems to be the method of choice. An appealing biomarker for clinical studies currently being considered is the use of a radiolabelled anti-AXL antibody to image AXL expression *in vivo* (Nimmagadda et al., 2014). This approach is highly amenable to immuno-PET imaging which could be incorporated into clinical studies for AXL inhibitors. One potential biomarker that has not been explored is the quantification of soluble AXL in blood serum samples of cancer patients. Soluble AXL (sAXL) is derived from proteolytic cleavage of the extracellular portion of membrane-bound AXL or is a product of alternative splicing (Verma et al., 2011). The biological significance of sAXL is unclear but may act to inhibit AXL signaling through binding and depleting its ligand GAS6 or, alternatively, act to concentrate GAS6 locally to activate AXL. Our collaborator Dr. David Bearrs and his group showed a significant increase in sAXL in advanced stages of pancreatic cancer patients compared to controls, with high/intermediate levels corresponding to poor outcomes (personal communication). We hypothesize that intermediate/high levels of sAXL and/or GAS6 will indicate metastasis in cancer patients and represent a novel biomarker for informing cancer treatments. Future studies are required to determine if sAXL is an appropriate biomarker to identify patients with advanced metastatic disease that will successfully respond to treatment with AXL inhibitors.

One outstanding question in the development of AXL inhibitors for clinical use is how does AXL inhibition impact cancer immunity? AXL and the rest of the

TAM family members are expressed by dendritic cells, macrophages, and immature natural killer cells of the immune system (Lemke and Rothlin, 2008). All TAM receptors function in the clearance of apoptotic cells and TAM knock out mice develop a plethora of autoimmune disorders. This is in part due to the normal function of AXL in suppressing inflammatory cytokine production in innate immune cells (Sharif et al., 2006). Loss of AXL in an inflammation-associated colorectal cancer model augments production of inflammatory cytokines that enables a tumor-promoting environment and increased tumor formation (Bosurgi et al., 2013). These observations raise the possibility of potential adverse effects of AXL inhibitors in the treatment of cancer. However, other studies provide evidence that inhibition of AXL signaling can enhance *in vivo* activity of natural killer cells against cancer cells and restrain cytokine production in tumor associated macrophages to suppress tumor growth (Paolino et al., 2014; Ye et al., 2010). These studies indicate AXL inhibitors can modulate immune cell function to promote antitumor immunity and underscore the importance of understanding the tissue and cell type specific functions of AXL in the tumor microenvironment to develop effective therapeutics.

The role of retinoids in cancer therapy

All-*trans*-retinoic acid (ATRA), an active metabolite of vitamin A, is an attractive anticancer therapy for the treatment of a variety of cancers because of its low toxicity and specific effects on inhibiting cell proliferation, inducing differentiation, and apoptosis (Connolly et al., 2013). Retinoid therapies are being

incorporated into the clinic as differentiation agents to target dormant cancer stem cells (Nasr et al., 2008; Reynolds, 2000). Our findings provide an alternative viewpoint on retinoid therapy and indicate the positive effects of retinoids may also be due to its role in blocking EMT. Currently ATRA derivative 13-*cis*-retinoic acid (13-cis-RA) is successfully used in the treatment of children with high-risk neuroblastoma to reduce the risk of recurrence after chemotherapy and stem cell transplantation and increase long-term survival (Matthay et al., 2009). However, ATRA is only routinely and effectively used for the treatment of cutaneous T cell lymphoma (Duvic et al., 2001) and acute promyelocytic leukemia (APL) (Bushue and Wan, 2010; Tallman et al., 1997). Given our findings that implicate RA in the elimination of disseminated cancer cells through the inhibition of EMT, why are retinoids not being used to treat a wider range of cancers? The antitumor activity of ATRA has been studied in several animal model systems and clinical trials but limited chemopreventative and therapeutic success has been observed in solid tumors (Connolly et al., 2013; Schenk et al., 2014). This may be partly due to the frequent epigenetic silencing of retinoic acid receptor β (RARβ) in tumors (Glasow et al., 2008; Lotan et al., 1995; Mehrotra et al., 2004; Sirchia et al., 2000), which may render treatment with ATRA ineffective. Re-expression of RARβ has been seen after treatment with epigenetic inhibitors (Schenk et al., 2012; Sirchia et al., 2002). Several studies have indicated that the combination of ATRA and epigenetic inhibitors may be an effective strategy to restore the therapeutic effects of ATRA in RARβ silent cancer cells (Pili et al., 2012; Qian et al., 2005; Raffoux et al., 2010; Sirchia et al.,

2002). These studies suggest the optimized use of retinoids to treat solid tumors may be dependent on evaluation of RARβ expression in solid tumors and combination treatment with epigenetic inhibitors. Future studies are required to evaluate the effectiveness of RA to both eliminate disseminated cancer cells through the inhibition of EMT and promote differentiation of cancer stem cells in cancer patients with solid tumors.

Overall, our approach to develop an *in vivo* EMT reporter to identify effective inhibitors of EMT has revealed novel signaling pathways regulating EMT during development and yielded a potent small molecule inhibitor of EMT. Additionally our screen provided rational therapeutic targets for melanoma treatment and a new viewpoint on retinoid therapy that that will help inform therapeutic strategies in the clinic.

References

Angelillo-Scherrer, A., Burnier, L., Flores, N., Savi, P., DeMol, M., Schaeffer, P., Herbert, J. M., Lemke, G., Goff, S. P., Matsushima, G. K. et al. (2005). Role of Gas6 receptors in platelet signaling during thrombus stabilization and implications for antithrombotic therapy. *J Clin Invest* **115**, 237-46.

Bosurgi, L., Bernink, J. H., Delgado Cuevas, V., Gagliani, N., Joannas, L., Schmid, E. T., Booth, C. J., Ghosh, S. and Rothlin, C. V. (2013). Paradoxical role of the proto-oncogene Axl and Mer receptor tyrosine kinases in colon cancer. *Proc Natl Acad Sci U S A* **110**, 13091-6.

Bushue, N. and Wan, Y. J. (2010). Retinoid pathway and cancer therapeutics. *Adv Drug Deliv Rev* **62**, 1285-98.

Byers, L. A., Diao, L., Wang, J., Saintigny, P., Girard, L., Peyton, M., Shen, L., Fan, Y., Giri, U., Tumula, P. K. et al. (2013). An epithelial-mesenchymal transition gene signature predicts resistance to EGFR and PI3K inhibitors and identifies Axl as a therapeutic target for overcoming EGFR inhibitor resistance. *Clin Cancer Res* **19**, 279-90.

Connolly, R. M., Nguyen, N. K. and Sukumar, S. (2013). Molecular pathways: current role and future directions of the retinoic acid pathway in cancer prevention and treatment. *Clin Cancer Res* **19**, 1651-9.

Duvic, M., Hymes, K., Heald, P., Breneman, D., Martin, A. G., Myskowski, P., Crowley, C., Yocum, R. C. and Bexarotene Worldwide Study, G. (2001). Bexarotene is effective and safe for treatment of refractory advanced-stage cutaneous T-cell lymphoma: multinational phase II-III trial results. *J Clin Oncol* **19**, 2456-71.

Glasow, A., Barrett, A., Petrie, K., Gupta, R., Boix-Chornet, M., Zhou, D. C., Grimwade, D., Gallagher, R., von Lindern, M., Waxman, S. et al. (2008). DNA methylation-independent loss of RARA gene expression in acute myeloid leukemia. *Blood* **111**, 2374-7.

Holland, S. J., Pan, A., Franci, C., Hu, Y., Chang, B., Li, W., Duan, M., Torneros, A., Yu, J., Heckrodt, T. J. et al. (2010). R428, a selective small molecule inhibitor of Axl kinase, blocks tumor spread and prolongs survival in models of metastatic breast cancer. *Cancer Res* **70**, 1544-54.

Lemke, G. and Rothlin, C. V. (2008). Immunobiology of the TAM receptors. *Nat Rev Immunol* **8**, 327-36.

Lotan, R., Xu, X. C., Lippman, S. M., Ro, J. Y., Lee, J. S., Lee, J. J. and Hong, W. K. (1995). Suppression of retinoic acid receptor-beta in premalignant

oral lesions and its up-regulation by isotretinoin. *N Engl J Med* **332**, 1405-10.

Mahadevan, D., Cooke, L., Riley, C., Swart, R., Simons, B., Della Croce, K., Wisner, L., Iorio, M., Shakalya, K., Garewal, H. et al. (2007). A novel tyrosine kinase switch is a mechanism of imatinib resistance in gastrointestinal stromal tumors. *Oncogene* **26**, 3909-19.

Matthay, K. K., Reynolds, C. P., Seeger, R. C., Shimada, H., Adkins, E. S., Haas-Kogan, D., Gerbing, R. B., London, W. B. and Villablanca, J. G. (2009). Long-term results for children with high-risk neuroblastoma treated on a randomized trial of myeloablative therapy followed by 13-cis-retinoic acid: a children's oncology group study. *J Clin Oncol* **27**, 1007-13.

Mehrotra, J., Vali, M., McVeigh, M., Kominsky, S. L., Fackler, M. J., Lahti-Domenici, J., Polyak, K., Sacchi, N., Garrett-Mayer, E., Argani, P. et al. (2004). Very high frequency of hypermethylated genes in breast cancer metastasis to the bone, brain, and lung. *Clin Cancer Res* **10**, 3104-9.

Mollard, A., Warner, S. L., Call, L. T., Wade, M. L., Bearss, J. J., Verma, A., Sharma, S., Vankayalapati, H. and Bearss, D. J. (2011). Design, synthesis and biological evaluation of a series of novel Axl kinase inhibitors. *ACS Med Chem Lett* **2**, 907-912.

Nasr, R., Guillemin, M. C., Ferhi, O., Soilihi, H., Peres, L., Berthier, C., Rousselot, P., Robledo-Sarmiento, M., Lallemand-Breitenbach, V., Gourmel, B. et al. (2008). Eradication of acute promyelocytic leukemia-initiating cells through PML-RARA degradation. *Nat Med* **14**, 1333-42.

Nimmagadda, S., Pullambhatla, M., Lisok, A., Hu, C., Maitra, A. and Pomper, M. G. (2014). Imaging Axl expression in pancreatic and prostate cancer xenografts. *Biochem Biophys Res Commun* **443**, 635-40.

Paolino, M., Choidas, A., Wallner, S., Pranjic, B., Uribesalgo, I., Loeser, S., Jamieson, A. M., Langdon, W. Y., Ikeda, F., Fededa, J. P. et al. (2014). The E3 ligase Cbl-b and TAM receptors regulate cancer metastasis via natural killer cells. *Nature* **507**, 508-12.

Pili, R., Salumbides, B., Zhao, M., Altiok, S., Qian, D., Zwiebel, J., Carducci, M. A. and Rudek, M. A. (2012). Phase I study of the histone deacetylase inhibitor entinostat in combination with 13-cis retinoic acid in patients with solid tumours. *Br J Cancer* **106**, 77-84.

Qian, D. Z., Ren, M., Wei, Y., Wang, X., van de Geijn, F., Rasmussen, C., Nakanishi, O., Sacchi, N. and Pili, R. (2005). In vivo imaging of retinoic acid receptor beta2 transcriptional activation by the histone deacetylase inhibitor MS-275 in retinoid-resistant prostate cancer cells. *Prostate* **64**, 20-8.

Raffoux, E., Cras, A., Recher, C., Boelle, P. Y., de Labarthe, A., Turlure, P., Marolleau, J. P., Reman, O., Gardin, C., Victor, M. et al. (2010). Phase 2 clinical trial of 5-azacitidine, valproic acid, and all-trans retinoic acid in patients with high-risk acute myeloid leukemia or myelodysplastic syndrome. *Oncotarget* **1**, 34-42.

Reynolds, C. P. (2000). Differentiating agents in pediatric malignancies: retinoids in neuroblastoma. *Curr Oncol Rep* **2**, 511-8.

Schenk, T., Chen, W. C., Gollner, S., Howell, L., Jin, L., Hebestreit, K., Klein, H. U., Popescu, A. C., Burnett, A., Mills, K. et al. (2012). Inhibition of the LSD1 (KDM1A) demethylase reactivates the all-trans-retinoic acid differentiation pathway in acute myeloid leukemia. *Nat Med* **18**, 605-11.

Schenk, T., Stengel, S. and Zelent, A. (2014). Unlocking the potential of retinoic acid in anticancer therapy. *Br J Cancer* **111**, 2039-45.

Sharif, M. N., Sosic, D., Rothlin, C. V., Kelly, E., Lemke, G., Olson, E. N. and Ivashkiv, L. B. (2006). Twist mediates suppression of inflammation by type I IFNs and Axl. *J Exp Med* **203**, 1891-901.

Sheridan, C. (2013). First Axl inhibitor enters clinical trials. *Nat Biotechnol* **31**, 775-6.

Sinha, S., Boysen, J., Nelson, M., Secreto, C., Warner, S. L., Bearss, D. J., Lesnick, C., Shanafelt, T. D., Kay, N. E. and Ghosh, A. K. (2015). Targeted Axl inhibition primes chronic lymphocytic leukemia B cells to apoptosis and shows synergistic/additive effects in combination with BTK inhibitors. *Clin Cancer Res* **21**, 2115-26.

Sirchia, S. M., Ferguson, A. T., Sironi, E., Subramanyan, S., Orlandi, R., Sukumar, S. and Sacchi, N. (2000). Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor beta2 promoter in breast cancer cells. *Oncogene* **19**, 1556-63.

Sirchia, S. M., Ren, M., Pili, R., Sironi, E., Somenzi, G., Ghidoni, R., Toma, S., Nicolo, G. and Sacchi, N. (2002). Endogenous reactivation of the RARbeta2 tumor suppressor gene epigenetically silenced in breast cancer. *Cancer Res* **62**, 2455-61.

Tallman, M. S., Andersen, J. W., Schiffer, C. A., Appelbaum, F. R., Feusner, J. H., Ogden, A., Shepherd, L., Willman, C., Bloomfield, C. D., Rowe, J. M. et al. (1997). All-trans-retinoic acid in acute promyelocytic leukemia. *N Engl J Med* **337**, 1021-8.

Tolero Pharmaceuticals. (2015). TP-0903, vol. 2015: [Internet]

http://www.toleropharmaceuticals.com/TP-0903.html.

Verma, A., Warner, S. L., Vankayalapati, H., Bearss, D. J. and Sharma, S. (2011). Targeting Axl and Mer kinases in cancer. *Mol Cancer Ther* **10**, 1763-73.

Wang, X., Saso, H., Iwamoto, T., Xia, W., Gong, Y., Pusztai, L., Woodward, W. A., Reuben, J. M., Warner, S. L., Bearss, D. J. et al. (2013). TIG1 promotes the development and progression of inflammatory breast cancer through activation of Axl kinase. *Cancer Res* **73**, 6516-25.

Wu, X., Liu, X., Koul, S., Lee, C. Y., Zhang, Z. and Halmos, B. (2014). AXL kinase as a novel target for cancer therapy. *Oncotarget* **5**, 9546-63.

Ye, X., Li, Y., Stawicki, S., Couto, S., Eastham-Anderson, J., Kallop, D., Weimer, R., Wu, Y. and Pei, L. (2010). An anti-Axl monoclonal antibody attenuates xenograft tumor growth and enhances the effect of multiple anticancer therapies. *Oncogene* **29**, 5254-64.